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ULTRACENTRIFUGAL STUDIES ON SERUM AND SERUM FRACTIONS

BY

KAI O. PEDERSEN

Printed in Sweden by
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To the Memory
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PREFACE.

The essential parts of this work have been carried out at the Institute of Physical Chemistry at the University of Upsala during the period 1939 to 1944.

To the head of the Institute, Professor The Svedberg, I wish to express my sincere gratitude for his kind interest in my work and for much advice during the last fifteen years. I am also greatly indebted to him for all the facilities of the Institute which he has placed at my disposal.

I am very grateful to Professor Arne Tiselius and to my colleagues at the Institute for many stimulating discussions. My thanks are also due to Drs. Sven Gard, Jan Waldenström and Gösta Widström for much help in the collection of the different samples of blood and for many valuable discussions about serum problems, and to Professor Carl Drucker for making several determinations of specific volumes.

Mr. Evald Hellman has carried out, together with his staff, the greater part of the calculations on the experimental data, and I am thankful to him for many discussions relating to my experiments.

Part of the expenses connected with this work have been defrayed by grants from the Nobel Foundation, the Rockefeller Foundation and the Wallenberg Foundation.

The author wishes to express his very best thanks to Dr. D. F. Cheesman, Stockholm, for the linguistic revision of this work.

The publication of this thesis in English was assisted by the British Council.

Upsala, January 1945.

Kai O. Pedersen.

Abbreviations.

UC = Svedberg and Pedersen, 'The Ultracentrifuge', Oxford 1940.
UZ = " " " , 'Die Ultrazentrifuge', Dresden and Leipzig 1940.
s_{20} is given in Svedberg units, $1\ S = 1 \cdot 10^{-13}$ c.g.s. units.
D_{20} is given in units of 10^{-7}.
Δn = the difference in refractive indices between the protein solution and the corresponding buffer solution is in the text and tables given in units of 10^{-5}.

Explanation to the Centrifuge Diagrams.

A detailed description of centrifuge diagrams is to be found in UC p. 287 ff. or UZ p. 258 ff.

Unless otherwise stated the conditions were as follows:
Speed of centrifuge: 59,000 r.p.m.
Mean centrifugal field: 250,000 times gravity.
Thickness of cell: 12 mm.
On the sedimentation diagrams:
Ordinates Z in μ ($1\ \mu = 0{\cdot}001$ mm.).
Abscissae: z in mm. At meniscus $z = 0$.
t = time of exposure in minutes after reaching full speed.
b = distance between cell and scale in mm.
c = concentration of sedimenting high-molecular substance(s) as calculated from the sedimentation diagrams.
A = 'albumin' peak.
F = fetuin peak.
G = globulin peak.
X = 'X-component' peak.

The heavy curve in the diagrams represents the experimental curve. When a composite peak occurs, this has often been resolved (sometimes only tentatively) into the curves for the individual components as indicated by the dotted lines.

INTRODUCTION.

Serum, one of the main constituents of the blood, has long attracted the attention of research workers in chemistry, biology and medicine. Although much work has already been devoted to analysing this protein solution and to determining its constituents and their chemical and physical properties, much still remains to be done.

The introduction and development of the ultracentrifuge[1] by The Svedberg as a new research tool in protein chemistry has been of great advantage in the study of these substances. The ultracentrifugal examination of a number of naturally occurring soluble proteins revealed for the first time that, in solution, each of them generally existed as molecules all having the same well-defined size. The result greatly stimulated new research on proteins.

The development of the electrophoresis technique by Tiselius (1937, b, 1940, a, b)[2] has provided another new and valuable method for the investigation of protein solutions. It has been extensively used in the study of various normal, pathological and immune sera or other native fluids.

In order to give an indication of the significance of these and other methods used in the present work for the study of serum, it is necessary at this early stage to anticipate some of the results obtained.

When a protein solution, say human serum, is examined by ultracentrifugation or electrophoresis, the diagrams from the two methods show the presence of different components. In Fig. 1 a schematic representation of two such diagrams has been given. Each peak shown on the diagrams corresponds to a component in the ultracentrifugal or electrophoretical sense. Thus all substances having the same sedimentation properties, or rate of electrophoretic migration, are referred to as one component. An 'ultracentrifugal component' is not necessarily homogeneous in electrophoresis and vice versa, or in any other

[1] For a detailed description of this instrument, its theory, construction, use, history, and results, see the monograph by Svedberg and Pedersen (1940) (henceforth referred to as UC (English edition) or UZ (German edition)).

[2] See also the monograph by Abramson, Moyer and Gorin (1942).

method, but it may consist of a mixture of chemically individual components with identical sedimentation properties. In a similar manner an apparent 'electrophoretic component' may show two peaks in the sedimentation diagram. Thus the 20-component and the newly isolated fetuin (Pedersen, 1944, a, b) are both α-globulins according to electrophoresis, but the former has the sedimentation constant $s_{20} \sim 20$ and the latter $s_{20} \sim 3{\cdot}3$.

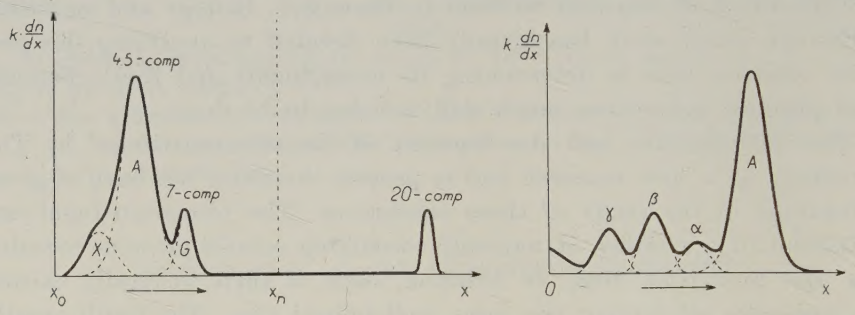

FIG. 1. Schematic representation of a sedimentation diagram (left) and an electrophoresis diagram (right) for normal human serum under standard conditions.

(The diagrams are pure constructions. In actual practice the ultracentrifuge cell is so short, that with the resolution shown for the X, A and G components, the 20-component would have settled to the bottom of the cell long before.)

The picture would more closely resemble a true sedimentation diagram if the bottom of the cell were placed at x_n. The arrows in the figure indicate the direction of sedimentation and electrophoretic migration respectively.

The designation of the various components has been made in the following way. The peaks marked A consist mainly of albumin, that with G of globulin, that with X of X-component and those with α, β and γ of α-, β-, and γ-globulin, respectively, (according to the classification by Tiselius (1937, c)). The additional nominations in the sedimentation diagram, 4·5-comp., 7-comp., and 20-comp., refer to the sedimentation constants, $s_{20} \sim 4{\cdot}5$, 7, and 20. The sedimentation constant for the X-component is variable and dependent upon the density of the solution (see p. 29 ff.).

At the moment it is not possible to give the exact relationship between the ultracentrifugal and the electrophoretical components. As an approximation one may say that the X, A, G and 20-components in the sedimentation diagrams correspond to β, A, γ and α in the electrophoresis diagram, when the same solution is investigated in the same concentration by both methods. In most sera other than human, the X-component sediments with about the same s_{20} as the albumin and its presence cannot be revealed by the sedimentation diagram. *It must be emphasized, therefore, that the A-peak in the ultracentrifuge*

diagrams may often contain some protein components other than the albumin. The relationship between the various fractions obtained by salt precipitation and by various other methods will be discussed in Chapter II.

The ordinates in the diagrams are proportional to dn/dx, but as most of the proteins have specific refraction increments, dn/dc, from 0·0018 to 0·0020, we may, as a first approximation, replace $k\ dn/dx$ with $k_1\ dc/dx$. This implies that when the curve is integrated between two arbitrary x-values, the concentration difference between the corresponding two levels in the cell is obtained. This may also be expressed as follows, the area of the individual peaks in the diagram is proportional to the concentration of the corresponding component in the ultracentrifuge, or electrophoresis, cell. By determining the position of the peaks at different times during the experiment, s_{20}, or the electrophoretic mobility, u, may be calculated for the various components. The sedimentation, or electrophoresis, diagrams thus allow us to characterize the different components by their values of s_{20} or u and to determine their concentration from the area of the corresponding peak. When the concentration was thus determined from the area of the $(X+A)$- and the G-peaks, it was found that the ratio $\frac{\text{concentration of } (X+A)}{\text{total concentration}}$ increased with increasing protein concentration, while at the same time the ratio $\frac{\text{concentration of } G}{\text{total concentration}}$ decreased (Mutzenbecher 1933, a; McFarlane 1935, a). McFarlane found that the same phenomenon also occurred in mixtures of horse serum albumin and horse serum globulin prepared according to the classical salting out methods. The present author (1936—39) obtained similar effects with lactoglobulin + serum albumin and in several, but not all, other protein mixtures investigated.

In order to study the phenomenon in more detail, it was considered necessary to use as well-defined and pure proteins as possible. It has been a general experience, in Upsala also, (*cf.* UC pp. 374 and 375, or UZ pp. 338 and 340) that it is not an easy matter to prepare pure serum proteins, a result which has been wholly confirmed by the present investigation.

The definition of a 'pure protein' presents certain difficulties because of the close similarity in composition and properties of many of the proteins occurring together in nature. The usual criteria of organic chemistry, such as constant melting point, constant composition and

properties after repeated recrystallizations from different solvents[1], can usually not be applied here. (For a critical review and discussion of these problems *cf.* Pirie (1940)). At present it seems that the most sensitive proof of purity may be obtained from solubility measurements conducted in the right way. Very thorough investigations in this sphere have been made by Northrop and his collaborators in Princeton. (For a discussion of these questions see, for instance, Kunitz and Northrop (1938, a, b) and Butler (1940)).

Very often, however, an ultracentrifugal or electrophoretical investigation of the substance will immediately reveal the presence of more than one component. Very little material is needed for such an ultracentrifugal examination (5 to 20 mg.); it is, therefore, a great advantage when an ultracentrifuge is available for following experiments on the fractionation of protein systems. Electrophoresis is also very useful, but, at present more material is required for a single experiment than in ultracentrifugation, and it seems reasonable not to use this method before the purification has advanced somewhat.

From the remarks made above it is evident that the presence of a single homogeneous component in either the sedimentation or the electrophoresis diagram does not imply that the substance in question is pure. If it is 'pure' according to both methods the probability that the protein is also pure with respect to other methods is naturally much greater. In order to establish the purity of a given protein it is important that it be checked in as many ways as possible.

From the part of this investigation dealing with the preparation of the serum proteins present in some quantity, it seems that only the fetuin and a globulin with low solubility, and perhaps also an albumin, may be prepared in such a purity as to satisfy both the ultracentrifugal and the electrophoretical criteria. It seems likely, however, that solubility measurements, for example, may reveal that they are not pure. There is nevertheless the possibility that prolonged equilibration with salt solutions may bring about undesirable changes in the composition of the material investigated.

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[1] A number of investigations have shown that crystallinity is not necessarily an indication of purity when proteins are concerned. 'Of the various criteria of purity that are used and accepted in the study of large molecules crystallinity is the most misleading' (Pirie 1940). On the other hand crystalline precipitates are much easier to handle and therefore offer several advantages in the preparation and in the study of properties such as solubility.

If it be considered that one of the functions of the plasma proteins is to transport a number of different substances from one place in the body to another, it is perhaps easier to realize that these proteins may contain, or be 'contaminated' by, several different non-protein substances. Some of these may be so intimately connected with the functions of these proteins that their removal may completely change some of the biologically important properties of the proteins. Such a protein may perhaps be pure after this removal, but at the same time it must be considered a new and different substance from the original. On the other hand, some of the substances may merely be adsorbed and their withdrawal may actually signify a purification of the protein. They may often, however, be very difficult to remove quantitatively.

It appears to be very important in the preparation of pure proteins to work quickly and omit all unnecessary procedures. It is also imperative that the time of contact of the proteins with strong salt solutions be made as short as possible. In some cases the same is probably true for the salt-free solutions. In the selection of fractionation methods, such procedures should be favoured as lead to the goal in as few and short manipulations as possible. In other words each step in the fractionation must bring about a noticeable change in the relative amount of at least one of the components in the mixture to be fractionated. Otherwise the use of such a step cannot be justified, or, to cite Pirie (1940), 'It must, however, be emphasized that repeated crystallization often causes protein denaturation or modification and that nothing will be gained by recrystallization unless it is certain that unwanted constituents are being removed in the mother liquor. Ten recrystallizations have no bearing on a protein's purity if nothing has been removed by the last eight of them'.

In the present thesis, Chapter I is a study of some of the factors which influence the sedimentation diagram of serum, especially human. As a result of this work, some standard conditions have been proposed for the ultracentrifugal study of serum. Chapter II deals with the fractionation of various sera with ammonium sulphate. The main purpose has been to determine whether this method can be used to prepare some of the serum proteins in pure, or at least 'ultracentrifugally pure', condition. Attention has mainly been directed on the preparation of the globulins present in not too small amounts. Finally some sedimentation and diffusion measurements on a few of the somewhat purer proteins prepared are described in Chapter III.

Chapter I.

ULTRACENTRIFUGATION OF SERUM.

1. HISTORICAL.

The first investigation of serum in the ultracentrifuge was carried out by the author in the spring of 1930. The sedimentation was recorded by means of the light absorption method using short-wave ultraviolet light. As, however, the centrifugal field at that time was not more than $1 \cdot 10^5 \times$ gravity, only a single diffuse boundary could be observed. The movement of this boundary corresponded to a sedimentation constant between that for serum albumin and that for serum globulin.

At the end of 1930, von Mutzenbecher made a few further experiments with serum in the ultracentrifuge. One of these, where the scale method was applied, indicated that if this method was used and the centrifugal field could be further increased, a separation of albumin and globulin would probably be observed.

In the spring 1932, when an improved Svedberg ultracentrifuge was ready for routine work, Mutzenbecher (1933) started an investigation of normal serum, using the Lamm scale method for recording the sedimentation. He found that albumin and globulin gave two separate peaks in the sedimentation diagrams, thus allowing their respective concentrations to be calculated. He observed, however, that when the same serum was measured at different concentrations, the ratio albumin/globulin, calculated from the sedimentation diagrams, increased with increasing serum concentration. During his work, he was to a certain extent hampered by the rigidity of the scale method at that time, in so far as one and the same scale distance had to be used throughout a whole run.

In the next ultracentrifugal examination of serum by McFarlane (1935), the scale method was made more flexible by the introduction of the scale-projection system, which allowed the most suitable scale distance to be chosen for each exposure. Furthermore it was possible

to use even the shortest distances, thus allowing even undiluted serum to be investigated. By using this amelioration in the observation technique, McFarlane made a very thorough investigation of normal sera from man, horse, and cow, and of pathological human sera. Finally, he studied some 'artificial sera' prepared by mixing serum albumin and serum globulin. He verified von Mutzenbecher's observation that the relative amount of serum albumin, computed from the sedimentation diagrams, was greatest in undiluted serum and decreased with increased dilution of the serum, whereas the opposite was the case for the globulin, both in normal and 'artificial serum'. They were, however, not able to offer any reasonable explanation for this concentration effect.

Von Mutzenbecher found that besides the albumin and the globulin with the sedimentation constants[1] 4·5 and 6·8, normal human and horse serum also contained small amounts of substances having $s_{20} = 9$ and 17. A component corresponding to the sedimentation constant 9 has not as yet been prepared with certainty, and it is questionable whether such a component exists. The 17-component may, in some cases, be prepared by means of fractional sedimentation in high speed centrifuges of the Beams-Bauer type.

McFarlane noticed the presence of a component with a sedimentation constant between those for albumin and globulin and he named this the '*X*-protein'. It was generally most distinct in the concentrated human sera, but disappeared into the 'albumin' peak in the dilute ones. Although the concentration of the *X*-protein amounted to 20—30 per cent of the total protein, according to McFarlane, he did not succeed in isolating this substance, nor did he observe it in the albumin or globulin fractions obtained by precipitation with ammonium sulphate. The present author is, however, of a different opinion after a re-examination of McFarlane's sedimentation diagrams, especially those from the normal globulin fractions. Besides the normal globulin peak these show the presence of a slower moving component which McFarlane assumed to be serum albumin. According to the author's experience, serum albumin is not precipitated by half saturated ammonium sulphate unless the *p*H of the mixture is adjusted to 5 or

[1] Reduced to infinite dilution. All sedimentation constants have been given in Svedberg units (S). By unanimous vote at the conference on 'The Ultracentrifuge' held by the *New York Academy of Sciences* on November 14—15, 1941, it was agreed to propose that 10^{13} times the fundamental unit for the sedimentation constant (in the C. G. S. system) be called the Svedberg (S).

below. If ammonium sulphate is added to serum without any acidification, the albumin will not be precipitated in any appreciable amount before the sulphate concentration in the mixture corresponds to 60 per cent saturation or even more. Since McFarlane did not wash his precipitate a small amount of albumin might have been present in this in the form of mother liquid, but this cannot account for the large quantity of substance sedimenting slower than the normal globulin, in one case 43 per cent and in another 22. As will be shown later, however, a protein with properties in the ultracentrifuge similar to those of McFarlane's *X*-protein is precipitated by 50—60 per cent saturated ammonium sulphate.

The two investigations mentioned above, by von Mutzenbecher and by McFarlane, are the only ones so far published dealing with the centrifugation of serum in general. In addition, a few immune and pathological sera have been studied by Heidelberger and Pedersen (1937), Jersild and Pedersen (1938), Kabat (1939), and Kekwick (1939, 1940) (see also UC p. 395, or UZ p. 357). From time to time other sera, normal and pathological, have been examined in Upsala, and it has often been found that the results are difficult to explain solely on the basis of von Mutzenbecher's and McFarlane's researches. It would therefore seem desirable to have some of the problems in connection with the sedimentation of serum in the ultracentrifuge studied in more detail. At the same time it may be expected that other problems related to the proteins in serum may be solved.

2. GENERAL.

The Material. The human sera used in this investigation were obtained from normal subjects working at the Institute. In order to reduce the suspended lipids and the like, the blood was always drawn by venepunction in the postabsorptive period. It was collected directly in centrifuge vessels which had been boiled previously in a water bath; after cooling, the tubes were rinsed with a 2 per cent sodium chloride solution in order to minimize the risk of haemolysis. 100—250 ml. blood was generally taken at a time. It was allowed to clot at room temperature for 6 to 8 hours; it was then centrifuged in an angle centrifuge after which the clear serum was drawn off with a pipette. The serum collected in this way was centrifuged once more in order to remove the last traces of corpuscles. When it was to be stored for a short time only, it was kept at + 2 to + 4° C, otherwise it was placed in a refrigerator at — 8° C.

In cases where plasma was wanted instead of serum, the blood was added directly to a 3·8 per cent solution of sodium citrate. The addition was discontinued when

the blood amounted to 9 times the citrate solution. After cooling for a few hours, the citrate blood was freed from the corpuscles by two centrifugings.

From cow and calf the blood was taken directly from the carotid of the animal at the Upsala slaughter-house. It was generally allowed to clot for 6 to 8 hours or overnight and then treated similarly to the human serum.

A. Selection of Experimental Conditions.

In order to limit the number of centrifuge runs in a given investigation it is necessary to select standard protein and salt concentrations. These should be chosen in such a way that the sedimentation diagrams give as much information as possible about the different components in the serum. As human serum seems to be more sensitive to variations in the experimental conditions, it seems reasonable to adapt these especially to this liquid.

Von Mutzenbecher (1933) generally used Ringer solution or physiological saline in his work; the serum was either undiluted, diluted very little, or diluted about ten times or more. As a rule, 1 per cent sodium chloride was McFarlane's (1935) diluent, or his solutions were dialysed against this solvent. No standard protein concentration was employed, but he usually preferred to work with the serum diluted relatively little. In the undiluted serum, certain complications appeared in the scale pictures, when the change in the refractive index through the cell was too abrupt. On the other hand, the *X*-protein disappeared into the 'albumin' peak when the dilution was too high. Later the present author (Jersild and Pedersen (1938) and UC p. 398, or UZ p. 360) observed that the sedimentation diagram was extremely sensitive to small alterations in the salt concentration, and he finally found that by choosing a suitable concentration of salt the *X*-component could be sufficiently separated from the 'albumin' peak in the sedimentation diagram. Under these conditions, the concentration of each of the two components could be determined separately. Before discussing the salt effect in more detail some experiments showing the variation with concentration of the sedimentation diagram, the calculated concentration of the different components and their values of s_{20} will be described.

B. Determination of the Amounts of the Different Components from the Sedimentation Diagrams.

By using the scale method (Lamm, 1937 and 1940) to follow the sedimentation in the ultracentrifuge cell, the concentration of the

individual components[1] may be calculated from the sedimentation diagrams, provided that the resolving power of the ultracentrifuge is sufficiently high. A description of the use of this method and the calculation of the concentration from the curves has been given by the present author in UC p. 287 ff. (UZ p. 258 ff.). It was found at an early stage in the application of the scale method that in some cases, for instance with the serum proteins, the concentration (or Δn) calculated from the sedimentation diagram was *always* less than that determined on the solution before centrifugation (McFarlane, 1935) while in other instances correct values were obtained (Pedersen, 1936, a, b, and UC p. 374, or UZ p. 338). This discrepancy for the serum proteins between the measured and the calculated values of Δn has also been confirmed in the present investigation.

The values of Δn from the sedimentation diagrams (Δn_{calc}) often deviated quite considerably from those measured in the refractometer (Δn_{obs}). When $\Delta n_{\text{calc}}/\Delta n_{\text{obs}} < 0\,7$, a centrifugation of the solution in the air-driven centrifuge at 27,000 R.P.M. for some hours revealed, in all instances, the presence of inhomogeneous heavy material. The Δn_{obs} decreased also in this procedure, while Δn_{calc} often remained constant and $\Delta n_{\text{calc}}/\Delta n_{\text{obs}}$ generally increased. A few of the results obtained after the removal of the inhomogeneous heavy material by high-speed centrifuging are given in Table 45 in the later part of Chapter II.

By spinning the solutions in the air-driven centrifuge $\Delta n_{\text{calc}}/\Delta n_{\text{obs}}$ could only be increased to about 0·8—0·9. In many cases no improvement was obtained when the initial value of $\Delta n_{\text{calc}}/\Delta n_{\text{obs}} > 0{\cdot}7$. It is seen from Table 1, p. 20, that the value of $\Delta n_{\text{calc}}/\Delta n_{\text{obs}}$ appears to be independent of the protein concentration in the experiment.

A number of runs were carried out on serum which had been dialysed against 0·2 M-NaCl; some of the solutions were spun in the air-driven centrifuge, others were not; most experiments were carried out at 59,000 R.P.M., while a single experiment was made at 40,000 R.P.M., but they all gave $\Delta n_{\text{calc}}/\Delta n_{\text{obs}} \sim 0{\cdot}8$. Some runs on dialysed sera and on a globulin solution were also performed in the separation

[1] Component should be taken here in the 'ultracentrifugal' sense of the word. Thus all substances having the same sedimentation properties are referred to as one component. An ultracentrifugal component is not necessarily homogeneous in electrophoresis and any other procedure, but it may consist of a mixture of individual components with the same sedimentation properties.

cell (UC p. 152 or UZ p. 132), and the nitrogen content of the upper compartment was determined after the run had been completed. In these experiments, the centrifuge was spun one hour more than necessary for the removal of the 'albumin' peak from the upper compartment. The analysis showed that from 0 to 5 per cent of the initial nitrogen was still present in this part of the cell. Phosphorus determinations gave in some cases values three times higher for P/N in the upper compartment after the run than before, probably indicating a relative increase in the phosphatide concentration.

The exact values for the scale distance, b, and for the cell thickness, a, were also re-determined, according to UC p. 295, foot-note, (UZ p. 265, foot-note), and found to deviate insignificantly from the values used for the calculation of the concentration. As $\Delta n_{calc}/\Delta n_{obs}$ seems to be about 0·8 independent of the concentrations used in these experiments, the possible errors discussed by Bridgman and Williams (1942) cannot greatly influence Δn_{calc}.[1] The error discussed by these authors should also 'improve' the value of Δn_{calc}.

In the ultracentrifuge cell, the protein solution is always covered, except in the experiments with the separation cell, with a layer of pure paraffin oil for preventing evaporation from the liquid during the run. This oil was considered to be indifferent and harmless in connection with proteins. As it had to be supposed, however, that lipids were more or less associated with the serum proteins, there was a slight possibility that the lipid-serum protein complex might 'react' with the paraffin oil. A few 'test-tube' experiments showed this to be the case.

In the first experiment 4 ml. of serum was mixed with 2 ml. of paraffin oil and vigorously shaken. The two liquids were separated by centrifuging and Δn was determined for the serum solution, but no change was observed although some denatured material had collected between the two liquids.

In the next experiment, similar amounts of serum and oil were mixed, but not shaken. The tube was gently reversed a few times. By this procedure the Δn was decreased from 1282 to 1199. After 4 hours spinning in the air-driven centrifuge at 27,000 R.P.M. Δn was reduced to 1182, *i.e.* a total reduction of about 8 per cent.

In a final experiment with a new serum and with the same proportions between

[1] With concentrated solutions, sharp peaks and short scale distances the presence of such errors may be demonstrated by displacement of the camera objective (and the plate holder). In such cases, the concentration calculated for the component giving the sharp peak is too high, especially when the peak is near the meniscus or the bottom of the cell.

oil and serum, a few gentle mixings reduced Δn from 1517 to 1483. Some further occasional reversal of the vessel containing the mixture and standing at room temperature reduced Δn to 1249. The experiment was extended over several days with occasional gentle mixing, but the measurement of Δn from time to time gave in all cases 1249, *i.e.* a total reduction of about 18 per cent.

The above results made it natural to repeat the experiments with the separation cell, now, however, with the protein solution covered with paraffin oil. Two experiments were carried out, both with a protein concentration of about 2/3 of that mentioned above. In the first, the same length of run as above, 210 min., was used. In the second, this was reduced to 180 min. The nitrogen content of the upper compartment was reduced from 2·20 mg. N to 0·400 mg. N in the first run, and from 2·19 to 0·388 in the second. This corresponds to 18·2 per cent protein left in the first case (after 210 min.) or to 17·7 per cent in the second one (180 min.). The P/N ratio was the same in the upper compartment after the run as before. A difference was observed between the upper solutions with paraffin oil and without. In the former cases they appeared slightly turbid in certain illuminations, while in the latter they were completely clear.

The results of the above experiments thus show that the paraffin oil may be responsible for the greater part, if not all, of the 'normal discrepancy' between Δn_{obs} and Δn_{calc}. This result was found quite recently and it has not been possible, so far, to test the result directly with the scale method other than in a few instances, where Δn_{calc} was found to deviate only slightly from Δn_{obs} when the solution was run without paraffin oil. With paraffin oil, Δn_{calc} amounted to only about 80 per cent of Δn_{obs} for human serum. This means that in the case of serum the Δn_{calc} may have to be increased by 20—25 per cent. For most of the serum fractions the error is probably definitely smaller.

Since the ultracentrifuge runs have been carried out with the solution covered with paraffin oil, an error has been introduced in the calculation of the concentration of the individual components in the initial solution from the sedimentation diagram. It may be expected that only a total of about 80—90 per cent of the originally homogeneous components have revealed themselves in the diagram. The percentages of the individual components in the different fractions, calculated from the Δn for the solution before centrifugation and Δn calculated for the single constituents from the sedimentation diagrams are thus minimum values. Some of them may have to be increased by 10 to 25 per cent in order to give the actual concentrations of the individual components in the original solution. It is probable that the error involved will be different for the various com-

ponents, depending upon the nature of the lipids in the lipo-protein complex. In the fractionation experiments, where interest has mainly been directed on the globulin fractions, it seems likely that the error will generally be less than 15 per cent. In no case can the presence of the paraffin oil change the general trend of the experiments.

Many of the solutions contain smaller or greater quantities of inhomogeneous heavy material which disappears from the sedimentation diagram before the measurements start. This, together with the substances prevented from appearing in the diagram by the paraffin oil, is included under 'Inhomogen. material, not in sedimentation diagram' in the tables summarizing the results of the individual fractionation experiments. As, however, the values in this column are obtained as differences between the Δn of the original solution and the sum of the Δn's for the single components, they may be appreciably influenced by the fortuitous errors in the various measurements.

It seems reasonable to suppose that the lack of reliable sedimentation equilibrium measurements in the serum proteins may be due to errors in the determination of the concentration in the cell caused by the paraffin oil and the inhomogeneous heavy material. With other proteins, such as lactoglobulin, correct values for the sedimenting substance are obtained from sedimentation velocity runs, and sedimentation equilibrium measurements yield practically constant values for M all through the cell (Pedersen, 1936, a, b, and UC Tables 32 and 36).

C. Influence of Concentration.

Normal human serum and plasma were dialysed against a suitable buffer. After reaching equilibrium, the difference, Δn, between the refractive indices of the protein solution and the buffer was measured, and samples of the former were diluted with the latter to give the values of Δn required. Since some of the experiments were carried out with plasma, the buffer solution consisted partly of sodium citrate, the concentration of which was adjusted so as to give a convenient distance between the X-peak and the 'albumin' peak in the sedimentation diagram. The results obtained are shown in Fig. 2 and Table 1. The diagram shows that at the lowest concentration no indication of the X-component could be observed, but as the concentration increased this peak became more and more distinct.

The values for the concentration of the different components given in Table 1 are those calculated from the sedimentation diagrams, as-

TABLE 1. VARIATION OF s_{20} AND CONCENTRATION, MEASURED FROM SEDIMENTATION DIAGRAM, WITH CONCENTRATION OF SERUM OR PLASMA.

The serum (KOP $^{19}/_2$ 40) (or plasma (KOP $^{22}/_2$ 40)) was dialysed against the following buffer solution:

0·075 M. Na citrate + 0·0435 M-Na_2HPO_4 + 0·0027 M-NaH_2PO_4.

	Δn of solution before run	X-comp.			'Albumin'			Globulin			Calculated from diagram		$\frac{\Delta n_{calc.}}{\Delta n_{obs.}}$
		s_{20}	conc.	%	s_{20}	conc.	%	s_{20}	conc.	%	total prot. conc.	Δn	
Serum	400				3·91	1·24	92	5·99	0·11	8	1·35	255	0·64
	600	2·38	0·24	11	3·64	1·66	75	5·90	0·32	14	2·22	420	0·70
	800	2·45	0·66	21	3·40	2·06	65	5·48	0·45	14	3·17	599	0·75
	1000	2·25	1·05	28	3·10	2·31	63	5·16	0·34	9	3·70	699	0·70
Plasma	420	2·53	0·13	8	4·00	1·25	79	6·43	0·21	13	1·59	300	0·71
	500	2·74	0·32	14	3·90	1·61	72	6·13	0·30	14	2·23	421	0·84
	600	2·62	0·45	17	3·70	1·80	70	5·94	0·34	13	2·59	490	0·82
	710	2·61	0·66	21	3·53	2·09	68	5·69	0·34	11	3·09	584	0·82
	800	2·40	0·73	24	3·40	2·04	66	5·44	0·32	10	3·09	584	0·73
	900	2·23	0·90	25	3·17	2·32	64	5·09	0·38	11	3·60	680	0·76
	1000	2·18	1·06	27	3·10	2·48	64	5·10	0·34	9	3·88	733	0·73

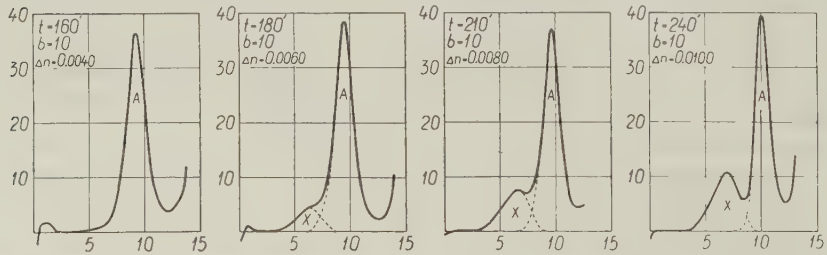

FIG. 2. Variation of sedimentation diagram of human serum with protein concentration.

The diagrams have all been reduced to the same protein concentration and scale distance, so that they are strictly comparable. For explanation of letters and other details, see p. 6.

suming the same refractive index increment, $\frac{dn}{dc}$, for all three components, viz.: $\frac{dn}{dc} = 0{\cdot}00189$ for the green Hg-line used in the centrifuge run and in the refractometer measurements. In calculating the total

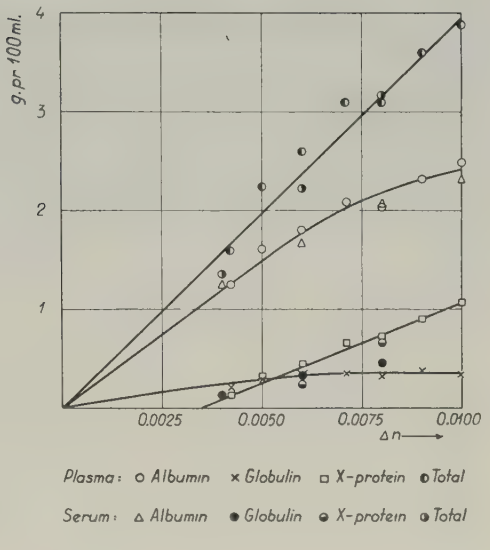

FIG. 3. Variation of the concentration of the individual components of human serum and plasma with Δn for the solution, as calculated for the sedimentation diagram.

In all cases, $\Delta n = 189$ has been assumed equivalent to 1 g. protein per 100 ml. solution.

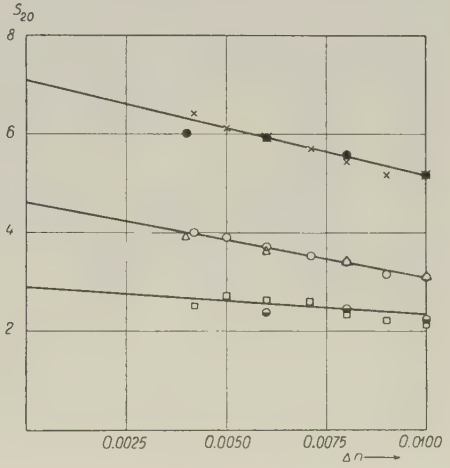

FIG. 4. Variation of s_{20} for the individual components of human serum and plasma with Δn for the solution.

protein concentration from the sedimentation diagrams no regard is taken to matter sedimenting faster than the serum globulin. Of this, the component with $s_{20} \sim 20$ corresponds to only a few per cent of the total protein; a small part consists of more or less ill defined matter sedimenting between the globulin and the 20-component. The remainder of the difference between Δn_{obs} and Δn_{calc} is undoubtedly due to the error produced by the paraffin oil as mentioned on p. 17—19.

In Fig. 3, the concentration calculated for the different components from the sedimentation diagram is plotted against the refractometric-

ally measured Δn. It is seen that the total protein concentration seems to be proportional to Δn_{obs}. For the individual components, however, this is not the case. For values of Δn higher than 500—600 the globulin concentration remains almost constant and the 'albumin' increases less than in the dilute solutions. The '*X*-protein' appears to start at about 350 and increases linearly. The occurrence and development of the *X*-peak with increasing protein concentration is best explained by assuming that the albumin and the globulin have reacted in some way with one another to form the '*X*-protein'. The figures for the 'albumin', globulin, and '*X*-protein' concentrations are not sufficiently reliable to allow detailed conclusions to be drawn about the exact proportions of albumin and globulin forming the '*X*-protein'. From the curve in Fig. 3, it is seen that the proportion is approximately 3 to 2. As, however, the molecular weight for albumin is about 70,000 and about 160,000 for globulin, it follows that the '*X*-protein' probably contains 3 to 4 times as many molecules of albumin as of globulin. For a further discussion see p. 31.

From the table it may be observed that the sedimentation constants vary considerably with protein concentration. In Fig. 4, s_{20} is plotted against Δn for the solution and for each component the best straight line is drawn. It is seen that the points fit the lines reasonably well and extrapolation to $\Delta n = 0$ gives for s°_{20} the values 2·9, 4·60 and 7·1 for the '*X*-protein', 'albumin', and globulin respectively, which values agree well with those obtained earlier in Upsala for human albumin and globulin.

The problem of the variation of the sedimentation constant with concentration has been theoretically studied by Burgers (1941—42) for spherical particles. From his investigation (Burgers, 1942) we may obtain the following formula:

$$s_c = s_0 \cdot \frac{1}{1 + 6{\cdot}875 \cdot c \cdot V}$$

where s_0 and s_c are the sedimentation constants at infinite dilution and at the concentration c respectively, c is given as grams of solute per cm^3 of solution and $V = 1/\varrho_p$, ϱ_p being the density of the particle. For non-spherical particles we may, at least as a first approximation, expect a similar formula to hold good. In this case, however, the value of the numerical constant must be increased. By plotting s_c as

ordinate against $c \cdot s_c$ as abscissa a straight line should be obtained, cutting the ordinate axis at s_0.

If we plot the values from Table 1 in a graph of s_c against $c \cdot s_c$, we will find that the points fit the straight lines almost as well as in the graph of s_c against c. The extrapolated values for s_0 (2·9, 5·0 and 7·6) do, however, seem somewhat too high. For the albumin and globulin the s_c values, calculated from the above formula, are higher than those experimentally determined.

Recently Lauffer (1944) has proposed a correction of the sedimentation constants, obtained at higher protein concentration, for the viscosity due to the protein. This method was tested in Upsala by the present author 8 to 10 years ago. In many cases $1/s_{20}$ was found to increase proportionally to the increase in viscosity with protein concentration (UC p. 275 or UZ p. 245). In some cases, however, it was found that the s_{20} 'corrected for viscosity' was almost as much higher than the s_{20} at infinite dilution as the uncorrected s_{20} was lower. The 'over-correction' found at that time may have been due to the presence of considerable amounts of inhomogeneous heavy material, which sedimented in the centrifuge to the bottom of the cell before the measurements were started, but in the viscosity determination caused an increase in η. It has not been possible to test Lauffer's proposal in the present investigation, but it may be desirable to do this in future work.

D. Influence of Salt.

As mentioned earlier, the appearance of the sedimentation diagram for human serum is highly dependent upon the concentration and nature of the salts present in the centrifuged solution. This salt effect was first observed accidentally by the author in 1938. Until then, the serum to be investigated in the ultracentrifuge was either diluted with 0·2 M-NaCl (or 1 per cent NaCl solution) or dialysed against a solution having the same composition. On a certain occasion, however, the author wished to make some centrifuge runs on normal human serum and as it happened that a reference run had been made shortly before on the buffer 0·2 M-NaCl + 0·02 M-NaH_2PO_4 + 0·03 M-Na_2HPO_4, the serum was diluted with a more concentrated stock solution of this buffer so as to give the salt concentration corresponding to the reference scale (in these runs no allowance was made for the salts already present in the serum). The appearance of the sedimentation diagram did not at all correspond to what was to be expected from McFarlane's investigation on normal human serum (see Fig. 5). The question was now whether the peculiar shape was due to some anomaly for that individual serum or whether it was caused by some salt effect.

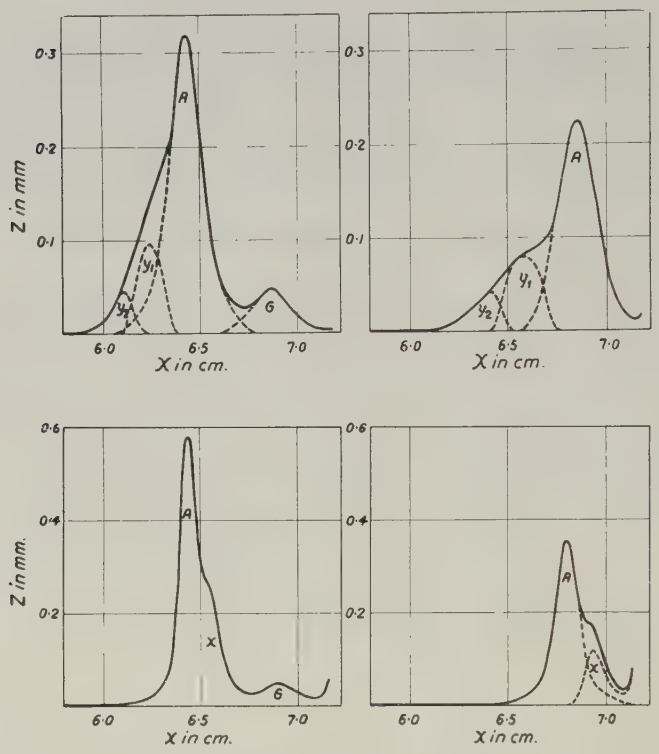

FIG. 5. The effect of salt on the sedimentation diagram for normal human serum (UC Fig. 143).

a. 1 vol. of serum diluted with 1 1/2 vol. of (0·33 M-NaCl + 0·033 M-NaH_2PO_4 + + 0·050 M-Na_2HPO_4).

b. 1 vol. of serum diluted with 1 1/2 vol. 0·33 M-NaCl.

Mean centrifugal force: 300,000 times gravity. Cell thickness: 12 mm. Mean temperature: 29° C.

	a		b	
	Left	Right	Left	Right
Time after reaching full speed (min.)	105	155	115	145
Scale distance in mm.	30	40	20	30

The composite peaks have been resolved according to the practice adopted earlier. *A* then represents the albumin, *G* the γ-globulin, y_1+y_2 and *X* the labile β-globulin. (As shown later in the text the classification earlier adopted is incorrect in Fig. 5 b where *A* should be exchanged for *X* and *vice versa*.

The same serum was therefore run under exactly the same conditions, except that it was now diluted with 0·33 M-NaCl. The result was that sedimentation diagrams similar to those of McFarlane were obtained (*cf.* Figs. 5 a and 5 b). Other normal human sera from different individuals gave similar results. A few preliminary experiments showed

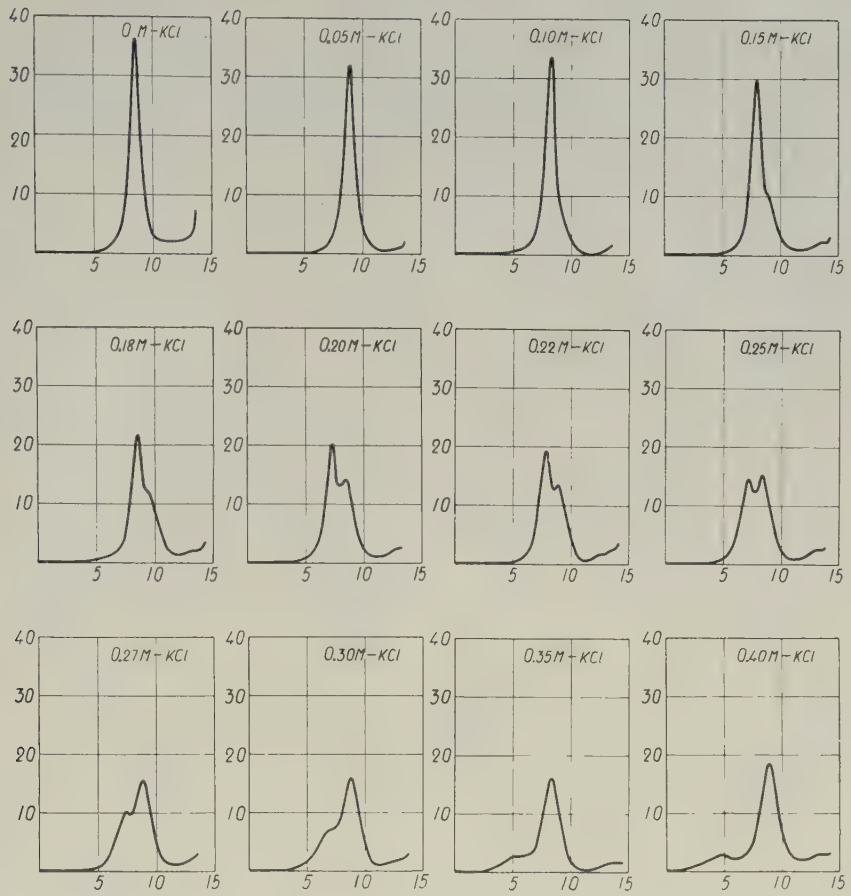

FIG. 6. The effect of varying the concentration of potassium chloride on the sedimentation diagram for normal human serum.

All diagrams recalculated to the same theoretical scale distance: 1 mm. Exposures taken 130 to 145 minutes after reaching full speed. Mean temperature: 27° C. Serum: KOP $^{10}/_{10}$ 1939. Serum concentration: 0·4 c_0.

a marked sensitivity to small variations in the salt concentration (UC p. 400, or UZ p. 360). It was here found that curves similar to those found with the above mentioned phosphate buffer could also be obtained with sodium chloride solutions by increasing the concentration of the added salt to 0·3—0·4 molar.

Different electrophoretically isolated fractions from human serum were also investigated with respect to the salt effect. It was found that the $(\beta+\gamma)$-globulin fraction contained one component with $s_{20} \sim 7$,

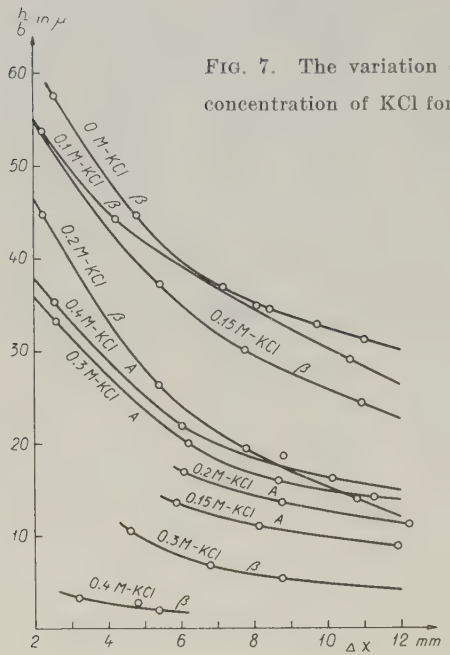

FIG. 7. The variation of the reduced height of the peak $\left(\frac{h}{b}\right)$ with concentration of KCl for different distances from the meniscus (Δx).

The curves are marked with the salt concentration and either A or β. For 0 and 0·1 M-KCl one curve only is given. In the higher concentrations two peaks could be obtained from the sedimentation diagrams, one corresponding to the albumin peak (A) and the other to the β-globulin. (Up to 0·2 M-KCl the β-peak is always higher than the A-peak.) Mean temperature, serum and serum concentration as in Fig. 6.

another component whose sedimentation constant varied with the salt concentration ($3 < s_{20} < 5$) and finally some inhomogeneous material. When the γ-globulin fraction was examined alone in the ultracentrifuge it showed mainly the normal globulin sedimentation with $s_{20} \sim 7$ independent of the salt concentration. It was concluded, therefore, that the labile component which earlier in this work has been called X-protein was a β-globulin (UC p. 400, or UZ p. 360). As, however, electrophoretically isolated β-globulin is very unstable, the experiments with the salt effect were continued with human serum.

Fig. 6 shows the sedimentation diagrams from a later series of experiments with potassium chloride added in concentrations varying from 0 to 0·4 molar. The diagrams given in Fig. 6 are chosen in such a way that the 'albumin' peak has sedimented almost the same distance in each case. The diagrams have all been reduced to the same theoretical scale distance of 1 mm. The figure shows clearly the very pronounced variation of the sedimentation diagram with the salt concentration.

When the influence of the nature of the salt was studied further, it was found that some salts already showed very pronounced effects

at low concentrations, while others were apparently not capable of affecting human serum. Simultaneous investigations on other sera, cow, horse, rabbit, and pig, did not reveal any salt effect.

For the study of the salt effect in more detail, it is not satisfactory merely to compare the appearance of the sedimentation diagram, but it is desirable to obtain some quantitative measure of the effect, which may make it possible to follow numerically the variation in the curves with changes in salt and concentration. For this purpose the reduced height of the 'albumin' (+ β-globulin) peak may be used in the following way. The heights of the peaks h (in μ) are divided by their scale distances b[1] (in mm.) and plotted against their distances from the meniscus Δx. Finally a smooth curve is drawn through the points. On

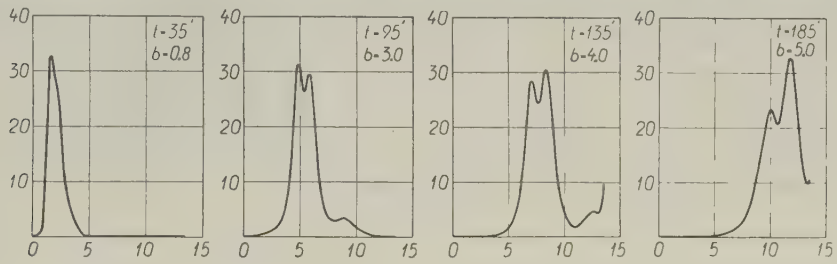

FIG. 8. The variation of the sedimentation diagram with time at a salt concentration of 0·25 M-KCl. Details as in Fig. 6.

this curve the values for $\frac{h}{b}$ are read, *e.g.* for every 2 mm. distance from the meniscus (see Fig. 7). In the cases where the peak is composite, each peak is treated separately (see Figs. 6 and 7). When no salt effect occurs, the curves for different salt concentrations will more or less coincide in the $\frac{h}{b} - \Delta x$ graph. If the curves are markedly displaced by changes in salt concentration, this indicates a salt effect. It is not always the same peak which is the highest throughout the run. It often happens that the β-globulin ('*X*-protein') peak has the highest value in the first part, and the 'albumin' peak in the last part of the run, as seen in Fig. 8.

For further investigation of the influence of the salt and a comparison of the results obtained with different salts, a new diagram (or a series of diagrams) may be constructed, where $\frac{h}{b}$ is plotted against the molarity of the solution for constant Δx, *e.g.* $\Delta x = 6$ or 8 mm. In

[1] b is the optical distance between the scale and the middle of the rotating column of solution. For further details see UC p. 287 (UZ p. 258).

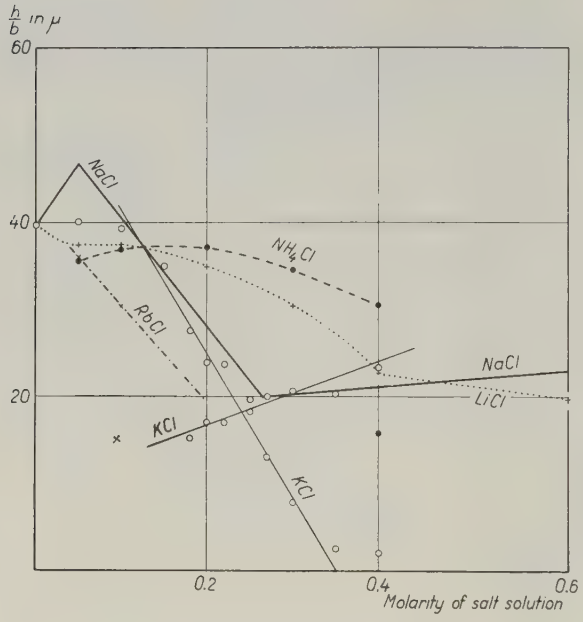

FIG. 9. The variation of the reduced height of the peak $\left(\frac{h}{b}\right)$ for $\Delta x = 6$ mm. with the concentration of the salt solution for different chlorides.

For the sake of simplicity, no experimental points have been plotted for NaCl, only the experimentally determined curve being given. Mean temperature, serum, and serum concentration, as in Fig. 6.

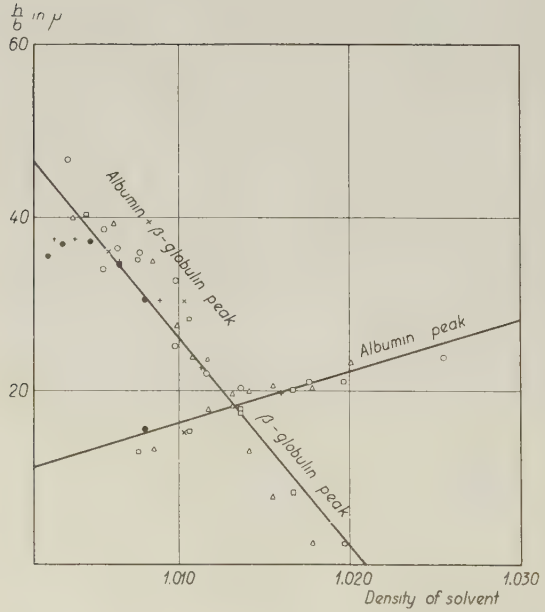

FIG. 10. The variation of the reduced height of the peak $\left(\frac{h}{b}\right)$ for $\Delta x = 6$ mm. with the density of the salt solution for different salts.

Mean temperature, serum, and serum concentration, as in Fig. 6.

Fig. 9 the results are shown of such experiments with ammonium, lithium, sodium, potassium, and rubidum chloride. It is seen from the curves that the variation in $\frac{h}{b}$ with concentration is much more pronounced in solutions of the last-named alkali chloride than in those of the first. In a further graph, Fig. 10, $\frac{h}{b}$ is plotted against the increase, $\Delta\varrho$, produced in the density of the solution by the different salts. It is quite evident from the position of points in the diagram that some relationship must exist between the height of the peak and the density of the solution. It is also seen that the two straight lines, the positions of which have been determined by the method of least squares, fit the experimental points as well as may be expected.

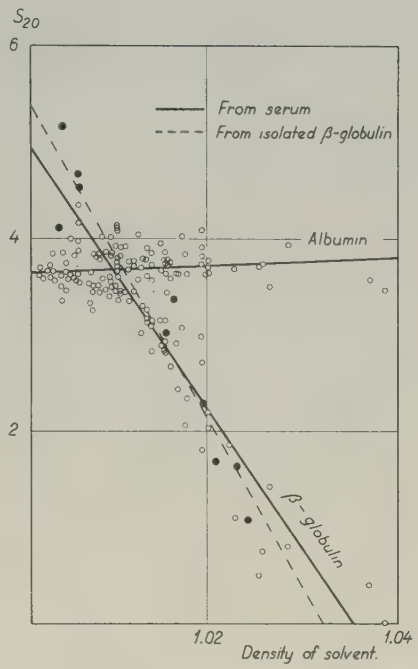

FIG. 11. The variation of s_{20} for albumin and β-globulin with the density of the solution.

⊙ refers to values obtained in runs with serum. • refers to values from runs on β-globulin ('*X*-protein'), isolated as described on page 30. Details as in Fig. 6.

The result in Fig. 10 made it natural to explain the effect as solely determined by the density of the centrifuged solution and as caused by a difference in the specific volumes of the albumin and the β-globulin. As, in the routine correction of the sedimentation constant, it is assumed that $V_{20} = 0{\cdot}749$, it follows that a substance with $V_{20} \leqslant 0{\cdot}749$ must show a drift in s_{20} with a change in the density of the solution. In Fig. 11 values of s_{20} for the 'albumin'- and the β-peaks have been plotted against $\Delta\varrho$ for a number of different salts (NH_4Cl, LiCl, NaCl, KCl, RbCl, CaCl, MgCl, $NaClO_3$, $NaNO_3$, $NaHCO_3$, NaSCN, $(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$, Na_2HPO_4, *etc.*). Experiments with saccharose and glucose have given results similar to those with neutral salts. Addition of small amounts of ether (1—2 per cent) has also produced some effect. In this case the β-globulin particles take up the greater part of the ether and their density decreases. From the appearance of Fig. 11 it might be supposed that the β-globulin would

show a negative sedimentation (*i.e.* sediment from the bottom toward the top of the cell) at values of $\varrho > 1{\cdot}04$. This implies that by using a suitable salt solution it should be possible to concentrate the β-globulin at the top of the cell while the rest of the proteins sediment towards the bottom, although at a lower rate. If the method was to be used preparatively, it was necessary to have such a density that for the negative sedimentations $s > 10$. If, on the other hand, the salt concentration was too high, the proteins might precipitate. It was finally decided to add $MgSO_4$ to about 0·45 saturation, this salt concentration being insufficient to produce an appreciable precipitate.

Ultracentrifugal Concentration of the β-globulin. 7 ml. of serum (KOP $^{13}/_{11}$ 43) was mixed with 5 ml. of saturated $MgSO_4$ and spun for about 20 hours at 18,000 r.p.m. in two large 'preparative cells' in one of the electrically driven ultra-

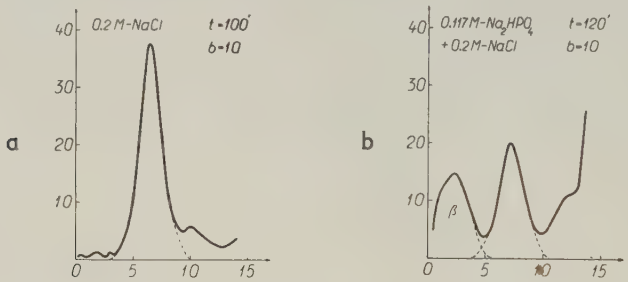

Fig. 12. Sedimentation diagrams from runs with a β-globulin ('X-protein') preparation from human serum.

centrifuges (temperature: 20° C). A distinct, oily, brown layer had collected at the top of the cell. When the cells were removed from the rotor, some mixing took place. The top layer was removed and dialysed against 0·2 M-NaCl. The ultracentrifuge diagram (Fig. 12 a) showed the presence of a single component with $s_{20} = 4{\cdot}53$. This value is very close to that which might be expected for β-globulin according to Fig. 11. In the case of the peak in Fig. 12a being due to β-globulin, one might expect that the appearance of the diagram would be much altered when the density of the solution was increased. By the addition of solid Na_2HPO_4, H_2O to 0·117 M-Na_2HPO_4, ϱ_{20} was changed from 1·0065 to 1·0230. The result is seen in Fig. 12 b. For the two peaks, the sedimentation constants 1·64 and 4·56 were obtained. The first value is in good agreement with the curve for β-globulin, while the second corresponds to s_{20} for albumin.

Similar separations of β-globulin were carried out on other samples of human serum (KOP $^{1}/_{12}$ 43, BF $^{1}/_{12}$ 43, BF $^{16}/_{2}$ 44). The concentration of the β-globulin was in all cases carried out in the air-driven centrifuge at 27,000 r.p.m. (length of run: about 6 hours). In some cases the top layer was again dissolved in half-saturated $MgSO_4$ and spun once more in the same way as above, whereby the *relative* concentration of the β-globulin was somewhat increased. The values for s_{20} found in the different

β-globulin solutions are plotted in Fig. 11, and the positions of all the lines in Fig. 11 have been determined by the method of least squares. For further details concerning the isolated β-globulin ('X-protein') see p. 166.

From the above mentioned experiments, it is clear that the tentative resolution of the composite peak in Fig. 5 b, made several years ago (UC Fig. 143) on the basis of McFarlane's interpretation of the sedimentation diagrams, is erroneous. The letter A should be substituted for X in the same diagrams and *vice versa*.

E. Discussion and Conclusions.

From the investigation just described and from other experiences gained from time to time, it was concluded that the most suitable concentration for the study of serum in the ultracentrifuge corresponded to solutions with $\Delta n \sim 600$—800. In the case of normal human serum this was equivalent to a dilution of one volume of serum with one to one and a half volumes of salt solution. In some instances, however, it has been found advantageous to study more concentrated sera (*cf.* p. 43).

With regard to the addition of salt to the serum, it was concluded that the most suitable density of the solution was $\varrho_{20} \sim 1{\cdot}015$—$1{\cdot}017$. Sedimentation runs carried out according to these stipulations will subsequently be referred to as having been made under standard conditions. The change produced in s_{20} for the β-globulin ('X-protein') will be referred to as the 'density effect'.

The experiments summarized in Table 1 have shown that the albumin and the globulin combine somehow with each other at the higher serum concentrations, whereby the 'X-protein' (or β-globulin) is formed. In the ultracentrifuge, the sedimentation of the β-globulin particle is highly dependent upon the density of the centrifuged solution as has been shown above. This means that the specific volume of this component must deviate considerably from $V \sim 0{\cdot}75$, the value for most proteins (UC, Table 48, see also p. 172). From the slope of the line for the isolated β-globulin in Fig. 11 a value of $V = 0{\cdot}969$ is calculated. In order to explain such a high value for V, it seems necessary to assume that the β-globulin particle contains definite amounts of lipids, in addition to the albumin and the globulin. It is also known from the investigations of Blix *et al.* (1941) that the β-globulin in human serum carries a great part of the serum lipids. According to their

analysis, β-globulin contains 8·7 per cent cholesterol and 10 per cent phospho-lipids (estimated from a lipid-P content of 0·40 per cent). From determinations of the phosphorus in ultracentrifugally isolated β-globulin by Mrs. B. Wikén (unpublished) it would appear that the content of phospho-lipids may be still higher. In addition to the lipids, Blix *et al.* found that the carbohydrate content of β-globulin was of the order of 10 per cent (calculated as dihexosamine), which means that the non-protein matter of the β-globulin particle may be of the order of 30 per cent or more.

If it be assumed that the β-globulin complex contains one globulin and three albumin molecules in addition to 30 per cent lipid + carbohydrate, the minimum particle weight must be about 500,000. It may be supposed that the β-globulin particle is not far from being spherical. For a spherical particle with $V = 0{\cdot}969$ and $s_{20} = 5{\cdot}9$ (the extrapolated value for β-globulin in water) the particle weight is calculated to be $\sim 1{\cdot}9 \cdot 10^6$ or about three times the minimum value. In the last figure, however, the water of hydration is included.

In a series of experiments still in progress it has recently been found by the present author (unpublished) that the concentration of the β-globulin component is increased when γ-globulin, prepared by salt precipitation, is added to the serum, and the serum concentration is kept constant. If the serum is not quite fresh no β-globulin peak can be observed neither before nor after the addition of the γ-globulin. This sensitivity of the β-globulin is probably due to the lipid part of the complex, where re-esterification and hydrolysis may take place, thus giving rise to new substances with different properties (*e. g.* lecithin → lysolecithin, *etc.*). Oxydation of the unsaturated fatty acid component of the phosphatides also seems possible.

From what has been said above it appears most likely to the author that the β-globulin ('*X*-protein') is a reversibly dissociable compound formed by albumin, globulin and some lipids with a total particle weight of the order of magnitude $1 \cdot 10^6$. Its slow sedimentation is due to the small difference between its density and the density of the solutions generally used.

At first sight one would expect that such a compound-formation in serum could be most easily demonstrated in electrophoresis by filling the lower part of the U-tube with a concentrated serum and using a slightly diluted serum as supernatant. The author has been kindly informed by Dr. H. Svensson, however, that he has found in a theo-

retical investigation (to be published shortly) that the boundary anomalies in such a case would, *inter alia*, be proportional not only to the difference in protein concentration between the two solutions, but also to the total protein concentration. This means that no clear cut result may be expected from such an experiment.

From ultracentrifugal runs of mixtures of human serum with various globulins it appeared that the *X*-components formed in these cases were highly dependent on the presence of the albumin; if the density of the solution was increased too much the *X*-peak vanished. The variation of the sedimentation of the *X*-peak with salt concentration also seemed to be less pronounced than in the case of the serum itself. This is perhaps an indication that the density of the 'artificial' *X*-protein (β-globulin) is higher than that of the native material.

The author has not observed, up to the present, any components showing the 'density effect' in sera other than human. As, however, it is known, for example from the work of McFarlane, that the increase in the globulin peak is less than proportional to the increase in serum concentration, while the opposite holds for the 'albumin' peak, it must be supposed that part of the globulin, in these sera also, combines with the albumin. It seems likely, however, that the densities of these compounds are such that they will be hidden in the 'albumin' peak.

3. ULTRACENTRIFUGATION OF DIFFERENT SERA AND PLASMAS.

A. Human Serum.

From time to time, a great many different human sera have been studied in the ultracentrifuge. References to the published papers have been given on pp. 12, 14 and 39. The influence of the dilution of the serum and the density of the centrifuged solution has also been discussed in the preceding pages. In Fig. 13 the sedimentation diagram is given from a run carried out under the standard conditions which were assumed to be the most suitable for investigating human serum. For the sake of comparison, the diagrams obtained by diluting serum from the same person with its supernatant are also given. The supernatant was in this case made by spinning the same serum undiluted in the high speed ultracentrifuge as long as necessary for transferring all

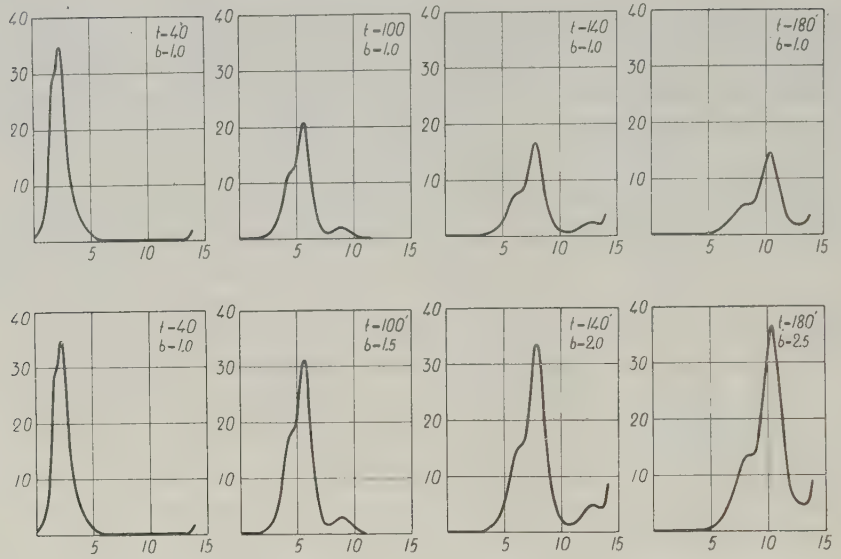

FIG. 13. Sedimentation diagram for normal human serum carried out according to the standard conditions.

Upper row: The appearance of the diagrams at constant scale distance.
Lower row: The routine appearance of the diagrams, where the scale distance has been adjusted to give approximately the same height of the peak.
Serum concentration: 0·4 $c_0 = 1$ volume serum diluted with 1 volume 0·2 M-Na_2HPO_4 and $^1/_2$ volume 0·1 M-Na_2HPO_4. Serum: KOP $^{10}/_{10}$ 39. Group: A. $\varrho_{20} = 1{\cdot}016$. Other conditions as in Fig. 6.

the albumin to the lower compartment of the separation cell (UC p. 152 or UZ p. 132). After concluding the run, the supernatant was collected in the upper compartment. It is seen from Fig. 14 that the sedimentation diagrams tell us much less about the serum in this case than in Fig. 13.

a. Normal Human Serum and Plasma from Individuals Belonging to Different Blood-Groups.

Until the spring of 1941, the author considered the curves seen in Fig. 13 as representative of the sedimentation diagrams for normal human serum under standard conditions. The examples given by McFarlane (1935, b) could also be interpreted in the same way. In one case only (FLH) had considerable deviations been found, in so far as that the amount of the labile β-globulin component (X-protein) was definitely higher than usual. The person in question, however, had been im-

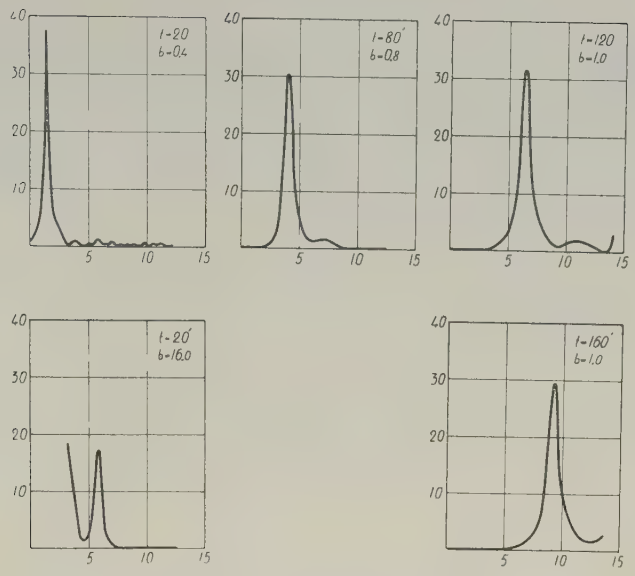

FIG. 14. Sedimentation diagram for normal human serum diluted with its supernatant.

Serum concentration: 0·4 c_0. Serum: KOP $^{19}/_2$ 40. The peak in the lower diagram to the left represents the 20-component. It may perhaps also be observed on the diagram just above, where the scale distance b is only $^1/_{40}$ of the value in the lower diagram.

munized against several different diseases, and it was thought that this would explain the divergence.

In connection with a study by Tiselius and Widström of the desiccation of serum and plasma for transfusion purposes (see Widström 1944), the author examined some of the dried samples. The first experiments showed that serum which had been lyophilized together with glucose gave sedimentation diagrams very much like those of the untreated serum. Those samples which were lyophilized without any addition gave less 'density effect' in the ultracentrifuge than the original serum. In this connection it may be of interest to note the following observation by Krejci (1942). She found that electrophoresis diagrams from pooled human citrate plasma, obtained after desiccation and reconstitution of the plasma, showed but a slight difference compared with those from the original sample, except that the β-globulin boundary was changed, the sharp β-peak on the descending side being reduced in height and sharpness.

Later on, defibrinated plasma, obtained from pooled, iso-haemagglutinin-free blood, was dried in the same way and examined in the

ultracentrifuge. The result was that no 'density effect' whatsoever could be detected. Experiments with a new, unpooled plasma showed that neither defibrination of the plasma with $CaCl_2$ nor filtration through a Seitz filter eliminated the 'density effect'. Finally, the original defibrinated plasma was investigated in the ultracentrifuge. This also showed no 'density effect'.

The whole problem was now somewhat obscure, the main difference between the two defibrinated plasmas being that one was obtained from pooled, citrated blood, the other from a single person. The pooled blood was collected from many persons belonging to the four blood groups A, B, O and AB, and was practically neutral as regards iso-haemagglutinins. The apparent difference between the two defibrinated plasmas was thus the presence or absence of the iso-haemagglutinins. In the former case the 'density effect' was observed; in the latter it had disappeared.

A number of new plasmas, from individuals belonging to the various blood-groups, were then examined in the ultracentrifuge. They all showed the 'density effect', although to different extents. The A-plasmas gave sedimentation diagrams similar to those in Fig. 13, whereas the B-plasmas, and the O-plasmas showed a much more pronounced β-globulin component (X-protein), the appearance of which was similar to that in the FLH serum mentioned in the beginning of this section. This serum was found also to contain α-agglutinins and must therefore belong to the B-group (or, in cases where the β-agglutinins had been destroyed during storage, to the O-group). The few AB-plasmas investigated showed only a small 'density effect'.

Other plasmas obtained by pooling blood from a not too small number of individuals belonging to the different ABO-groups gave results similar to those obtained with pooled defibrinated plasma. When the pooling was done with a small number of sera, a slight 'density effect' was sometimes observed. However, the plasmas from pooled blood belonging to a single group would also now and then show a small 'density effect'.

During the investigation of plasma from individuals belonging to the different groups, it was observed that the amount of the β-globulin component apparently decreased with time in so far as the part of the protein showing the 'density effect' declined. Comparison of serum and plasma simultaneously taken from the same individual gave as a result that when the density and serum concentration were equal, the

sedimentation diagrams were also alike. The serum, however, would retain its properties for several months when kept in the refrigerator at +2° to +4° C, whereas the plasma under similar conditions would deteriorate within a few weeks. Nevertheless, the serum does not remain unchanged even if it is kept at a low temperature. Thus the FLH-serum, which showed a prominent *X*-component, gave no 'density effect' after four years storage at −8° C.

In Fig. 15 some examples are given of sedimentation diagrams from plasmas and sera belonging to the groups B and O. It is seen that the '*X*-protein' dominates more in these diagrams than in Fig. 13.

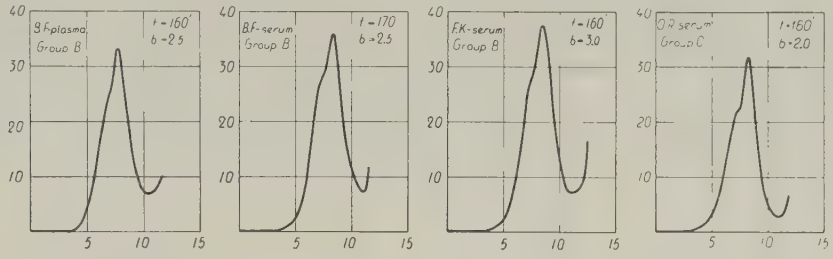

FIG. 15. Sedimentation diagrams for human sera and plasma from individuals belonging to the groups B and O.

The experimental conditions were the same as in Fig. 13, except that 'B.F.plasma' had been dialysed against a citrate-phosphate buffer with $\varrho_{20} = 1{\cdot}019$ and $\Delta n = 600$.

From the experiments just described it seemed, therefore, that some connection existed between the amounts of the labile '*X*-protein' and the haemagglutinins in the sera or plasmas. On the other hand, it was very difficult to conceive that the bulk of the '*X*-protein' could have any relationship with the agglutinins. In order to throw some light on the problem a number of runs were carried out with the separation cell (UC p. 152 or UZ p. 132). In these experiments, the change of the agglutinin titer in the upper compartment of the cell was determined after different time intervals. It was found that both agglutinins sedimented together with the 20-component. When both were sedimented in the same run, as with O-serum, a slight difference was observed in s_{20} between the α- and β-agglutinin, but this was probably due to experimental errors. Fractionation experiments with ammonium sulphate also showed that most of the agglutinins were precipitated between 0·37 and 0·45 sat. Am_2SO_4, *i.e.* not in the fractions containing the bulk of the labile '*X*-protein'.

A further number of sera belonging to the different blood-groups were investigated late in 1941 and at the beginning of 1942. Among these, an A-serum gave sedimentation diagrams very much like those in Fig. 15. On the other hand, O- and B-sera were found which showed no, or only very slight, 'density effects'. In Fig. 16 a sedimentation diagram from such a serum is given.

These new sera were also examined under electrophoresis and compared with the earlier 'normal' sera. It was not possible, however, to

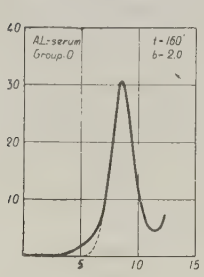

FIG. 16. Sedimentation diagram for a normal human serum, showing only a small 'density effect'.

Experimental conditions as in Fig. 13. The dotted line indicates the normal appearance of the albumin curve.

find any correlation between the sizes of the labile β-globulin peak in the sedimentation diagram and in the electrophoresis diagram.

The whole question at present seems rather obscure and nothing definite can be said about the cause for the absence or presence of the labile β-globulin.

As a pure speculation it may be explained for instance in one of the following manners.

1) The compound formation between albumin, globulin, lipids, *etc.*, takes place in different ways in those sera which show the 'density effect' and in those which do not. — Most of the animal sera show no such effect. Their albumin and globulin, however, undoubtedly combine with lipids, *etc.*, to form *X*-components similar to those demonstrated in human serum, but sedimenting together with the 'albumin'.

In this connection it may be of interest to note that in a few cases with rather concentrated sera an extra '*X*-component' has been observed between the labile β-globulin and the normal albumin peak (*cf.* Table 2, p. 48 (serum SL $^{26}/_{5}$ 38) and Fig. 22, p. 50).

2) In the agglutination process, β-globulin is adsorbed by the blood corpuscles-agglutinin complex in a manner similar to the binding of lipids to specific precipitates found by Horsfall and Goodner (1935—36) (see p. 45). — It may act as a complement in the agglutinin reaction.

3) The presence or absence of the β-globulin as a high-molecular lipid-protein complex is determined by some lipase in the blood. The concentration of this enzyme (or its activators, or inhibitors) is very different in the different bloods. In the mixing the factors favouring the disappearance of the labile β-globulin predominate.

In this connection, it may be mentioned that the β-globulin, isolated by centrifugation as described on p. 30, is very unstable and loses its ability to show the 'density effect' within some days, while the plasma, and especially the serum, retain this property much longer.

4) The amount of the labile β-globulin is determined in some way by the nutritional condition or habits of the individual. Thus, the sera which have been followed for several years showed perhaps a tendency to contain somewhat less labile β-globulin around 1942, when the nutritional situation here in Sweden was at its lowest level.

b. Pathological Sera.

A survey of the ultracentrifugation of pathological sera has been given by McFarlane (1935, c). A number of special pathological sera have been studied from time to time by von Mutzenbecher (1933, b), Jersild and Pedersen (1938), Kekwick (1939, 1940) and by the present author in collaboration with different physicians (*cf.* v. Bonsdorff *et al.* (1937, 1938), Packalén (1939), Bing (1940), Waldenström (1944 a, b), and Lehmann (1944), see also UC p. 400 or UZ p. 360).

A more detailed discussion of the pathological sera lies outside of the scope of this paper. It may be mentioned, however, that some of the sera investigated in collaboration with Dr. Waldenström were exceedingly rich in β_2-globulin according to electrophoretical measurements (50 to 70 per cent of the total protein), but the pathological protein sedimented as a 20-component (see Fig. 17).

It is seen from Fig. 17 that the pathological component is dominant in the sedimentation diagram. In order to obtain correct curves for

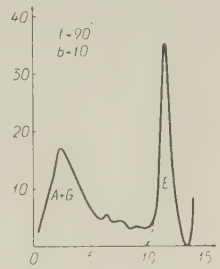

FIG. 17. Sedimentation diagram for a pathological serum from a patient (EAA) with hyperglobulinemia.

1 volume of serum diluted with 19 volumes of 0·2 M NaCl. Speed of the centrifuge: 40,000 R.P.M. Centrifugal field: 116,000 *g*. Cell thickness: 12 mm.

the 20-component it was necessary to dilute the serum 8 times more than usual, and it was found advantageous to run the centrifuge at 40,000 R.P.M. instead of at 59,000 R.P.M. When the serum was run under standard conditions the albumin and the globulin gave an almost normal sedimentation diagram, but the 20-component moved as a 'Schlieren' through the scale-image.

The presence of a single protein in large amounts in some of the pathological sera may sometimes offer possibilities of isolating and purifying this substance in an easier manner than usual for the serum proteins. See p. 122.

B. Other Sera.

Normal mammalian sera, other than human, have been studied in the ultracentrifuge on several occasions by von Mutzenbecher (1933, a), McFarlane (1935, b), Heidelberger and Pedersen (1937) and the present author. Immune sera have been examined by McFarlane (1935, c), Heidelberger and Pedersen (1937), Kabat and Pedersen (1938), Kabat (1939), and especially by Kekwick and Record (1941). The sedimentation diagrams from the normal sera were rather like those from normal human serum (in 0·2 M-NaCl), although some quantitative differences could be observed. In the immune sera an increased amount of one of the components was often observed. Most frequently the 'normal' globulin, with $s_{20} \sim 7$, was augmented, but in some cases the enlargement took place in the 20-component. Thus the antibody of the antipneumococcus horse sera was associated with the 20-component, while diphtheria antitoxic horse sera were found to contain two antitoxins associated with β- and γ-globulins respectively, and both sedimenting with $s_{20} \sim 7$. However, the antibody of the human antipneumococcus serum was associated with the normal globulin. Hence it is seen that one and the same antigen in some species gives rise to antibodies corresponding to the 'normal' globulin with $s_{20} \sim 7$ and in others to antibodies with $s_{20} \sim 20$. Within the same species, different antigens may produce antibodies of different molecular weights and electrophoretic properties.

Svedberg and Andersson (1938) have found that the sedimentation diagrams from sera belonging to the classes *Reptilia*, *Amphibia*, and *Pisces* were similar to those obtained from the higher classes of the vertebrates, whereas that from *Cyclostomata (Lampetra fluviatilis)* was distinctly different. In the last-named serum two components with $s_{20} = 3{\cdot}5$ and $s_{20} = 12$ predominate.

The author has tried to produce a 'density effect' in the sera from horse, cow, pig, rabbit, and sheep, but without success. Apart from most human sera such an effect has so far only been found in serum and plasma from chicken.

Shortly before the war, M. E. Adair and G. S. Adair started an examination in Upsala of maternal and fœtal serum from sheep and sera from lamb of different ages and from foal. The investigation, which was discontinued by the war, was planned to include both electrophoretical experiments and ultracentrifugal runs (in collaboration with the author). The sedimentation diagrams from fœtal serum showed great differences when compared with those from maternal and lamb sera. Thus in the former case the normal globulin with $s_{20} \sim 7$ was absent and the 'albumin' peak was of a composite nature somewhat reminiscent of the diagrams for 0·22 to 0·27 M-KCl in Fig. 6. At that time it was assumed that the asymmetry was caused by the presence of labile β-globulin. As a result of the investigations described later in this paper we know that it originates in the presence of fetuin in the fœtal serum.

According to a private communication, Svedberg and Andersson followed several years ago the changes taking place in the sedimentation diagrams from serum from chicken embryo during incubation. They found that the globulin with $s_{20} \sim 7$ did not appear until shortly before the hatching was completed. The 'albumin' peak was asymmetrical and gave low values for s_{20}. This may be explained by the presence of fetuin.

a. Sera from Calf and from Cow's Fœtus.

Before the fractionation experiments with calf serum were started, some ultracentrifugal runs were made on sera from calves of different ages. To begin with, 3 volumes of serum were diluted with 2 volumes of salt solution prior to the examination in the ultracentrifuge. The sedimentation diagrams showed one predominant peak which, in the case of serum from a newly born calf, toward the end of the run became asymmetrical or at least deviated markedly in shape from the normal probability curve (Fig. 18). Experiments with salt solutions of different concentrations did not disclose any 'density effect'. Later on, undiluted sera were used for the runs and the presence of fetuin was sometimes revealed by an extra peak close to that of the albumin, but it also happened that it was manifested only by the broad shape of the albumin peak, as shown in the last diagram of Fig. 18.

From the value of the sedimentation constants it was also evident that the 'albumin' peak could not be due exclusively to normal albumin. For the concentrations in question one would expect $s_{20} \sim 3{\cdot}7$ for the

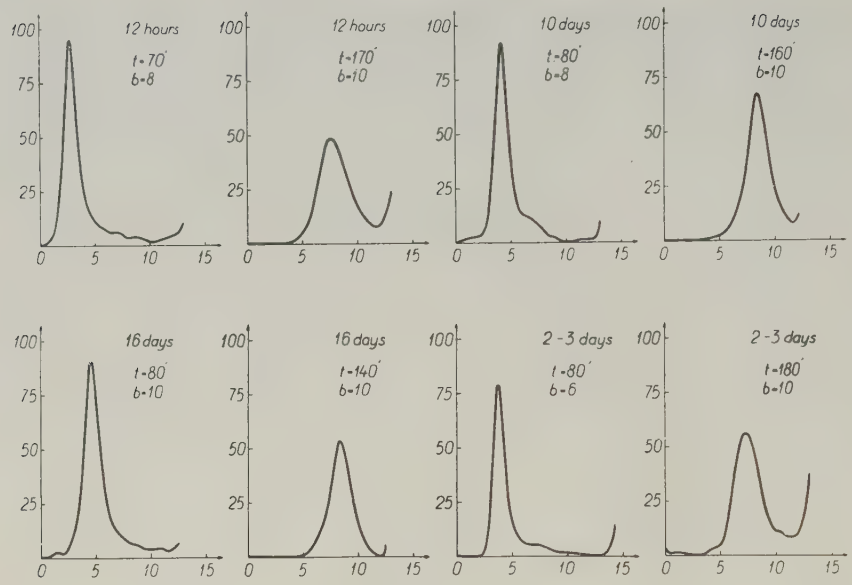

FIG. 18. Sedimentation diagrams for sera from newly born calves of different ages. (12 hours, 10 days, 16 days and 2 or 3 days.)

Serum concentration in the first 6 diagrams, 3 vol. serum diluted with 2 vol. 0·2 M-Na_2HPO_4; in the last two diagrams undiluted serum (same as used in Fractionation Experiment No. 7). All diagrams have been reduced to the protein concentration one per cent.

albumin peak. The following average values for s_{20} were found at the serum concentrations 0·6 c_0 and 0·4 c_0:

Age of calf:	12 hours	10 days	16 days	(>1 year)
s_{20} for $c = 0{\cdot}6\ c_0$	3·0	3·4	3·7	3·7
s_{20} for $c = 0{\cdot}4\ c_0$	3·2	3·9		4·0

When the relative amount of fetuin is higher in the serum, as for instance from the fœtus, its presence is easily detected in the sedimentation diagram, even in the cases where the serum has been diluted before the run.

In Fig. 19 the sedimentation diagrams are shown for three different concentrations of the same fœtal serum as used in Fractionation Experiment No. 17 (p. 110).

The experiments in this section have shown that it is difficult to observe the presence of a substance like fetuin except when its rela-

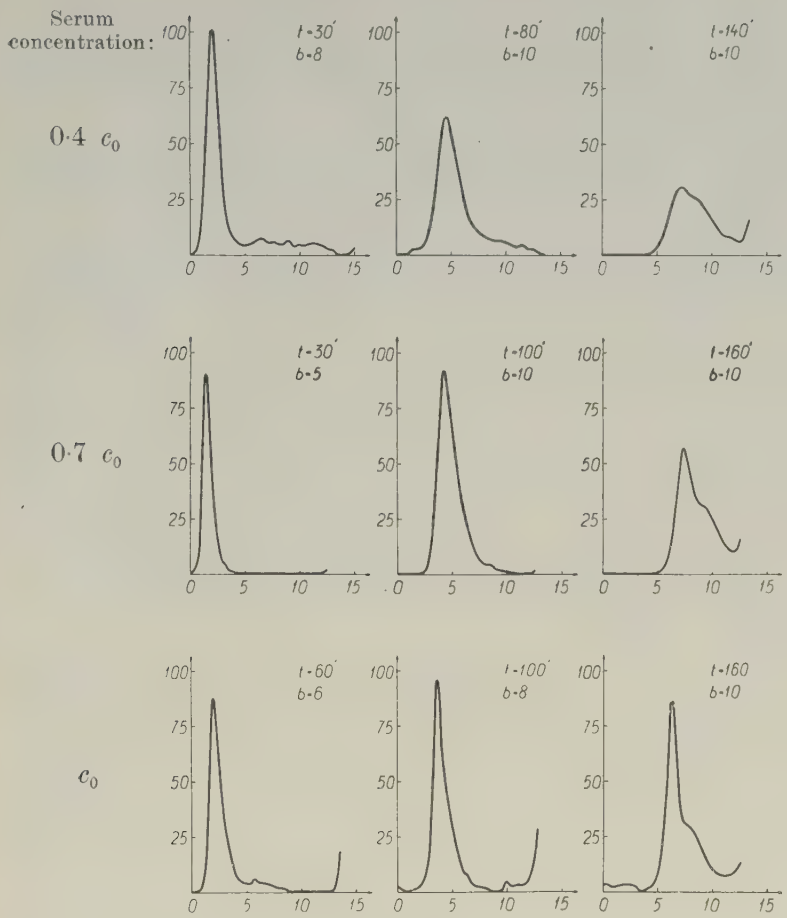

FIG. 19. Sedimentation diagrams for fœtal serum from cow.
All diagrams have been reduced to the protein concentration one per cent.

tive concentration is of the same order as that of the albumin. The detection of components not present in large amounts is favoured, however, by the use of relatively concentrated solutions.

C. Extraction of Lipids from Serum and Serum Proteins.

The problems connected with the lipids in serum have aroused much interest among immunologists and protein chemists, and much valuable information has been gained, but this still seems to constitute one of the fields in serum chemistry that is in urgent need of more investiga-

tion. The information in the literature about the lipid composition of sera is rather conflicting even when the analyses have been carried out by the same method.

The largest number of individual sera studied in one investigation was 66 male human subjects. For these sera Page *et al.* (1935) found the following values expressed in mg. per 100 ml. (figures after the ± sign indicate standard deviations): total lipids 735 ± 216, total cholesterol 232 ± 62, free cholesterol 82 ± 17, phosphatides 181 ± 71 and neutral fats 225 ± 137. According to Kirk (1938) human plasma (20 samples) contained a total of 145 mg. phosphatide per 100 ml., of which 19 mg. was lecithin, 68 mg. cephalin and 58 mg. other insoluble phosphatide (probably sphingomyelin). In addition to this Kirk found an average of 42 mg. cerebrosides, but he admitted that individual variations were enormous (0 to 167 mg. per cent). Brückner (1941) found, however, that no cerebroside could be detected in plasma or serum. Thannhauser *et al.* (1939) observed the following phosphatide values in normal human serum: lecithin from 50 to 200 mg., cephalin from 50 to 130 mg. and sphingomyelin from 15 to 35 mg. Later Artom (1941) found a total phosphatide content of 152 ± 16 mg. per 100 ml. whereof 30 ± 9·5 mg. was cephalin and 122 ± 14 mg. choline phospholipids (lecithin and sphingomyelin). The different methods used for determining the individual phosphatides have been criticized by Ramsay and Stewart (1941) who observed much higher values for the sphingomyelin content of whole blood than usually given in the literature.

The methods, most commonly used for removing the lipids from serum, are based upon the technique of Hardy and Gardiner (1910) using alcohol (or acetone) and ether at low temperature. The essential point is to keep the temperature down as long as the slightest amount of water is present. For different variations in the technique reference should be made to Young (1922), Hartley (1925), Hewitt (1927), Sorensen (1930), Horsfall and Goodner (1935) and Blix (1941).

A considerable part of the lipids may, according to McFarlane (1942), be removed when serum and ether are shaken together and the mixture is frozen below −25° C. After thawing, a large part of the lipids is dissolved in the ether phase and may be separated together with this. By repeating the procedure about $^2/_3$ of the lipids may be extracted.

By a third method (Macheboeuf and Tayeaux, 1941) serum or the protein solution to be delipidated is first mixed with a suitable potassium or sodium soap at a *p*H of 7 to 8. By this procedure the fatty acid displaces the lipid attached to the protein and the lipid may now be removed with ether in the cold. By adjusting the *p*H to about 5 the fatty acid may be extracted from the protein with ether. The authors claim that no denaturation of the protein occurs and that

the delipidated globulin, for instance, is much more soluble in dilute salt solutions (*cf.* also Putnam and Neurath 1944).

In 1925 Hartley found that some of the properties of delipidated sera were changed and others remained unaltered. Thus the specific precipitin reaction failed to appear when the lipids were removed from both the antigen and the corresponding antiserum. However, he had reason to believe that the antigen and antibody united, but that this antigen-antibody complex was soluble. This view was supported by the fact that diphtheria toxin united with the diphtheria antitoxin in the antiserum freed from its lipids. In this case also no flocculation occurred in the neutral zone. It was also found that the antigenic properties of normal sera were not lost when the lipids had been extracted.

Horsfall and Goodner (1935) found that the type specific agglutinin and precipitin reactions disappeared when the lipids had been removed from the Type I antipneumococcus horse serum, whereas these reactions were much reduced in the case of rabbit serum. The extracted sera were, however, still capable of protecting mice against fatal infection with Type I Pneumococci. The normal agglutinating and precipitating properties of the antisera could, however, be restored by the addition of minute amounts of lecithin or cephalin to the horse or rabbit antisera respectively. In their next work Horsfall and Goodner (1936, a) showed that the different mammals could probably be divided into two groups, each requiring its special phosphatide for the precipitin reaction. To the lecithin group belonged horse, man, mouse, cat, dog, goat, and probably also cow, whereas the cephalin group included rabbit, guinea pig, rat, and sheep. In a few other respects members within the same group showed the similar immunological properties, but behaved differently from the other group.

In a subsequent publication, the same authors (1936, b) demonstrated that the total lipid content of the specific precipitates was entirely independent of the protein content (they observed variations from 4 per cent to 51 per cent by weight of the specific precipitates). This quantity seems to be governed by the laws of adsorption and thus varies with the initial lipid content of the reaction mixture. Finally, Horsfall and Goodner (1936, c) analysed the specific precipitates for their contents of the individual lipids. The specific precipitates from horse and rabbit showed one very characteristic difference in their phosphatide composition, in so far as the first mainly contained an

amino phosphatide (probably cephalin) while the second contained a non-amino phosphatide which they supposed to be lecithin. This is quite remarkable and indicates that these substances are selectively adsorbed by the two specific precipitates respectively. The horse serum contained, in their experiment, almost twice as much phosphatide as the rabbit serum, but the ratio lipid amino-nitrogen/lipid nitrogen was almost the same in each case. Two other characteristic differences may be mentioned. Almost one fourth of the total lipid content of the two sera consisted of neutral fat, while the presence of this could not be detected in either specific precipitate. The sera contained rather little free cholesterol, whereas one third of the lipids in the specific precipitate consisted of this substance.

From Horsfall and Goodner's investigations we may draw the conclusion that if the lipids are an integral part of the antibody molecule or the antigen-antibody complex, they make up only a very small proportion of the whole particle. Most probably, the greater part of these substances is loosely bound to the protein molecule, but part is necessary to render the specific precipitate insoluble or sparingly soluble. From the electrophoretic findings of Theorell (1930), Mellander (1935). Blix, Tiselius and Svensson (1941) and Blix (1941) it seems reasonable to assume that at least the main part of the serum lipids is simply adsorbed and may be removed without causing much damage to these molecules. According to the investigations of Blix (1941), it seems, however, that the electrophoretic properties of the α-globulin are greatly changed when this protein has been deprived of its lipids.

From what has been found in this study it may be assumed that at least a part, perhaps only a small one, of the lipids constitute an integral part of the globulin molecules. When it is removed, the properties of the globulin molecule change from 'globular' to 'fibrous'. In the labile β-globulin lipid-protein complex (the '*X*-protein'), the lipids probably constitute a significant portion of the particle.

Experiments with the Ultracentrifuge. McFarlane (1935, b, e) studied human serum and serum globulin which had been delipidated by the method of Hardy and Gardiner according to the modification of Hewitt (1927). He found that the sedimentation diagrams for the same serum before and after the extraction showed great differences, in so far as in the latter case the components appeared polydisperse. He assumed this to indicate the first step of denaturation.

In 1938 the present author carried out some ultracentrifuge runs on human sera extracted with alcohol and ether at low temperature according to 'Method A' of Horsfall and Goodner (1935).[1] These experiments were carried out in order to determine whether the 'density effect' of the serum remained intact after the removal of the serum lipids. According to the results obtained this was not the case. It was then investigated whether an addition of suitable salts to the serum before extraction would protect the *X*-component. Small amounts of concentrated solutions of sodium chloride, sodium phosphate or magnesium chloride were added to the serum before the extraction. It appeared that addition of salt before extraction made the resulting dry powder less easily soluble in water and salt solutions. From Table 2 it is also evident that in this case much less material is observed in the sedimentation diagram per milligram of dissolved serum powder. When, however, it had been ascertained that the 'density effect' disappeared with the extraction of the lipids, these experiments were discontinued for the time being.

In connection with a recent investigation, it may be of some value to reconsider the experiments mentioned above, which are therefore summarized in Table 2 together with ultracentrifuge runs on the native material. The most striking change produced by the alcohol-ether extraction is the complete disappearance of the *X*-component from the sedimentation diagram. Another change is the strong increase generally observed in the inhomogeneous fraction sedimenting more rapidly than the globulin. This last material does also show a higher percentage in the extracted material than in the native, except in the case of a pathological serum with an initial high globulin content. Similarly the 'albumin'/globulin ratio is lower in the extracted sera than in the native ones, with the same exception as above. In the extracted material A/G seems to increase with increase in concentration.

If one compares the ratios:

$$\frac{\text{total protein concentration from sedimentation diagram}}{\text{mg. protein powder per g. solvent}}$$

one finds the highest values for the sera to which nothing has been added before extraction. This implies that more inhomogeneous heavy

[1] The extractions were made by Dr. F. L. Horsfall, Jr. The author is much indebted to Dr. Horsfall for much help and advice in connection with these experiments and for many valuable discussions.

TABLE 2. THE EFFECT OF DELIPIDATION

Material	mg. protein powder per g. solvent or serum concentration	Salt solution added[1]	mg. nitrogen per g. solution	Total protein concentration calculated from diagram
Normal serum FLH $^{13}/_{4}$ 38	0·8 c_0	Phosphate I		5·78
	0·4 c_0	" II	4·68	2·49
	0·4 c_0	0·33 M–NaCl	4·86	2·69
	0·2 c_0	0·25 M–NaCl	2·36	1·32
After extraction	60 mg.	0·20 M–NaCl		3·10
	15 mg.	do.	1·59	1·23
Phosphate added before extraction	56 mg.	do.		2·29
	56·5 mg.	Phosphate III	6·21	2·06
	21 mg.	0·20 M–NaCl		1·03
NaCl add. bef. extr.	60·7 mg.	Phosphate III		0·70
Normal extracted serum FLH $^{13}/_{5}$ 38	53 mg.	0·20 M–NaCl	13·3	2·52
	Same solution, phosphate added.	Phosphate IV		2·49
	106·6 mg.	0·20 M–NaCl	11·6	4·08
do. FLH $^{2}/_{6}$ 38	95.8 mg.	do.		4·66
do. KOP $^{2}/_{6}$ 38	99 mg.	do.	11·6	5·04
Normal serum SL $^{26}/_{5}$ 38	0·8 c	Phosphate I		5·47
	0·8 c_0	1 M–NaCl		5·69
After extraction	108·3 mg.	0·20 M–NaCl		5·32
	do.,phosphate added.	Phosphate III	12·8	5·37
Path. serum GH $^{23}/_{2}$ 38	0·09 c_0	Phosphate III	1·44	0·78
After extraction	64 mg.	0·20 M–NaCl		1·48
	16 mg.	do.		0·39
Serum 'albumin'		Phosphate V		1·58
After extraction	30·7 mg.	0·20 M–NaCl		2·06
$(\beta+\gamma)$-globulin		Phosphate V		0·94
After extraction	37·5 mg.	0·20 M–NaCl		0·66
	do.,phosphate added.	Phosphate III		0·32

[1] Phosphate I = 1 M-NaCl + 0·10 M-NaH_2PO_4 + 0·15 M-Na_2HPO_4
Phosphate III = 0·2 M-NaCl + 0·02 M-NaH_2PO_4 + 0·03 M-Na_2HPO_4
Phosphate V = 0·15 M-NaCl + 0·001 M-NaH_2PO_4 + 0·019 M-Na_2HPO_4

ON VARIOUS SERA AND SERUM PROTEINS.

X-component			'Albumin'			Globulin			Inhomogeneous material	
s_{20}	c	%	s_{20}	c	%	s_{20}	c	%	c	%
1·44	1·53	26	2·92	3·34	58	4·34	0·45	8	0·46	8
2·81	0·51	21	3·77	1·65	66	6·18	0·30	12	0·03	1
3·07	0·86	32	3·75	1·47	55	6·42	0·32	12	0·04	1
3·55	0·15	12	4·42	1·03	78	7·54	0·11	8	0·03	2
			3·65	2·44	79	5·29	0·66	21		
			4·47	0·67	54	6·51	0·23	19	0·33	27
			4·17	1·41	61	5·95	0·52	23	0·36	16
			4·20	1·17	57	5·97	0·47	23	0·42	20
			4·52	0·45	44	6·87	0·19	18	0·39	38
			4·02	0·54	77	6·15	0·13	19	0·03	4
			4·18	1·40	56	5·61	0·58	23	0·54	21
			4·14	1·48	59	5·72	0·61	25	0·40	16
			3·26	2·93	72	4·57	0·80	20	0·35	8
			2·75	3·26	70	3·56	0·65	14	0·75	16
			3·24	4·33	86	4·46	0·71	14	0	
1·41	1·06	19	2·86	3·36	62	4·52	0·59	11		
2·5	0·46	8								
2·28	1·78	31	2·88	2·70	47	4·50	0·71	13		
2·53	0·50	9								
			3·20	3·35	63	4·13	0·76	14	1·21	23
			3·33	3·12	58	4·41	0·80	15	1·45	27
3·35	0·03	4	4·37	0·41	52	6·65	0·34	44		
			4·51	0·80	54	6·50	0·37	25	0·31	21
			4·65	0·12	31	7·75	0·07	18	0·20	51
			4·19	1·58	100		0			
			4·28	1·09	53	6·48	0·47	23	0·50	24
β-globulin						γ-globulin				
4·36	0·28	30				6·32	0·66	70		
4·56	0·43	65				6·88	0·23	35		
5·69	0·20	63				7·43	0·12	37		

Phosphate II = 0·33 M-NaCl + 0·033 M-NaH_2PO_4 + 0·050 M-Na_2HPO_4
Phosphate IV = 0·2 M-NaCl + 0·036 M-NaH_2PO_4 + 0·063 M-Na_2HPO_4

material is formed when the different salts are added, or the serum is diluted, before the lipids have been removed.

Figs. 20 and 21 illustrate the effect of alcohol-ether extraction on two different human sera. The upper row in Fig. 20 shows the native serum in two dilutions. In the more concentrated, the *X*-component is clearly visible, whereas in the dilute serum it cannot be distinguished from the 'albumin' until in much later exposures. The lower row shows two dilutions of the same serum after the extraction. In the con-

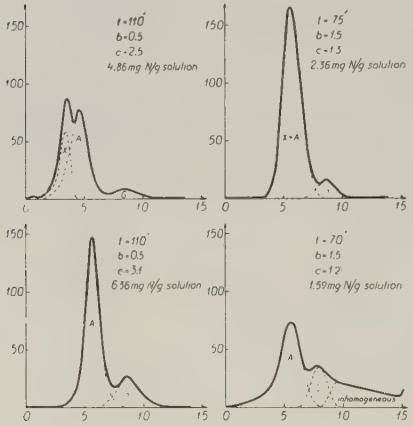

FIG. 20. The effect of alcohol-ether extraction on a human serum (FLH $^{13}/_4$ 1938).

Upper row: Normal human serum 2·5 and 5 times diluted with saline.

Lower row: Same serum reconstituted after extraction with alcohol-ether according to Horsfall and Goodner (1935).

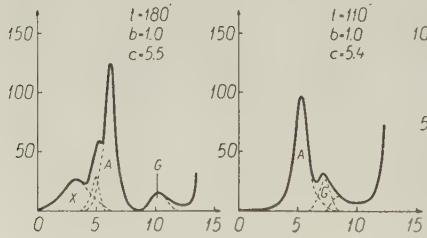

Fig. 21.

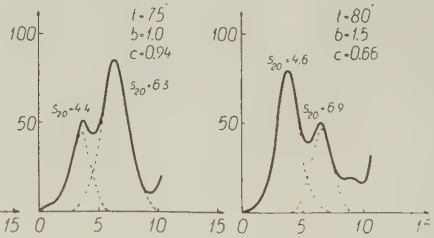

Fig. 22.

FIG. 21. The effect of alcohol-ether extraction on a human serum (SL $^{26}/_5$ 38).

Left: Serum before extraction. 4 vol. serum diluted with 1 vol. of 1 M-NaCl+ +0·10 M-NaH_2PO_4+0·15 M-Na_2HPO_4.

Right: Same serum after reconstitution, subsequent to extraction with alcohol-ether, and addition of salts to: 0·2 M-NaCl+0·02 M-NaH_2PO_4+0·03 M-Na_2HPO_4.

FIG. 22. The effect of alcohol-ether extraction on the $\beta+\gamma$-globulin fraction from normal human serum.

Left: Electrophoretically isolated $\beta+\gamma$-globulin in 0·15 M-NaCl+0·001 M-NaH_2PO_4+ +0·019 M-Na_2HPO_4.

Right: Same fraction reconstituted after extraction with alcohol and ether. Salt solution: 0·2 M-NaCl.

centrated solution both the globulin and the 'albumin' appear quite homogeneous, but in the dilute solution both show considerable inhomogeneity. The same behaviour was always encountered in the extracted sera (see also Fig. 21). The addition of extra salt on no occasion disclosed the presence of an *X*-component.

Certain other differences between the native and the extracted sera were also observed. The serum powders, where salts had been added before the alcohol-ether extraction, were generally less readily soluble than those where such an addition had not taken place. When a concentrated reconstituted serum was made from the powder, it often happened that after a while the whole solution formed a gel. Standing in an incubator overnight in most cases liquefied the gel.

There is a very distinct difference in the appearances of the bottom layer in the ultracentrifuge cell after runs on native and extracted serum. In the former case the consistency of the substance in the bottom of the cell is like that of syrup, while in the latter it may form a compact elastic gel, which may be removed from the cell as an entirety.

Extractions were also carried out on fractions obtained by electrophoretic separation of human serum in the apparatus of Tiselius (1937, b). Two such fractions were investigated: the albumin and one containing both β- and γ-globulin. The albumin fraction showed a single component only in the sedimentation diagram before the extraction. After the extraction the picture was quite changed, as seen from Table 2. In the case of the globulin fraction the proportion between the two components (in the centrifuge diagram) seemed to be reversed (Fig. 22).

Chapter II.

FRACTIONATION OF SERUM.

1. HISTORICAL.

During the last century numerous papers have appeared dealing with the fractionation of blood serum.[1] The purposes of all these investigations may be included under the following two headings.

1) The determination of the relative and absolute amount of the different proteins present in serum.

2) The preparation and study of the individual proteins.

Many studies have been carried out from the first point of view, in order to find the best methods for ascertaining the composition of a given serum or its deviation from the normal value. In some cases, the variation of the composition of the sera with species, age, and sex, has been investigated and considerable differences have been observed, especially between different species and between the adult and new-born animal.

A century ago, several authors recorded that dilution of serum with water and addition of a small amount of acid produced a precipitate in the mixture (*cf.* Scherer, 1841, p. 12; Zimmermann, 1846; Lehmann, 1850). Panum (1850—52) was the first, however, to realize that the precipitate was not albumin and he called the new protein serum casein. A decade later, Schmidt (1862), who discovered its importance in the clotting of blood, introduced the new name globulin for Panum's serum casein. Kühne (1868) was of a different opinion and called the CO_2-precipitate paraglobulin. After removal of the paraglobulin from the solution and addition of a small amount of acetic acid, a further precipitate could be obtained which he assumed to be a sodium albuminate. From a number of different experiments especially with cow serum, Heynsius (1869) came to the conclusion that the precipitate obtained by dilution of serum with water and acidifi-

[1] For more detailed reviews see Hammarsten (1902), Howe (1925), Geill (1938), and for the recent literature Cohn (1941) and Svensson (1941).

cation with CO_2 or acetic acid was the same as that gained by saturation of the filtrate from paraglobulin with sodium chloride. The clotting action of the precipitate was due to its contamination with an unknown active substance. Heynsius recommended the use of the name 'globulin' for these proteins. Weyl (1877) came to the same conclusion, namely that only one globulin was present in serum, and he called this serum globulin, a name which has since been generally used.

The methods hitherto described only yielded a part of those proteins which we now call serum globulins. The whole globulin fraction was precipitated, however, by two new methods. Hammarsten (1878) showed that when serum was saturated with magnesium sulphate, much more globulin was brought down than by the earlier methods, and that no albumin was carried down.[1] Later Kauder (1886) found that the same amount of globulin was precipitated by half saturation with ammonium sulphate.[2] The globulins separated in this way are usually called 'total globulin'.

Much discussion occurred in the literature as to whether the serum globulin and the serum albumin were well-defined uniform substances or mixtures. There was also some uncertainty as to whether the globulin, obtained by dilution and acidification of serum, was the same as that precipitated by saturation with $MgSO_4$ or by half saturation with Am_2SO_4. Towards the end of the last century, however, it was customary to call the proteins precipitated by half saturation with ammonium sulphate globulins, and those by full saturation albumins. It was assumed, however, that some other proteins, present in small amounts in the serum, were carried down, mainly together with the globulin.

At the beginning of this century it became evident that different properties were associated with different fractions of the globulin, and Fuld and Spiro (1900), after the suggestion of Hofmeister, introduced the name euglobulin for the globulin which could be precipitated by dialysis, whereas the other part was called pseudoglobulin. According

[1] Denis (1859) had found that serum albumin alone was left in solution when blood plasma was saturated with magnesium sulphate. The $MgSO_4$ method seems not to have been used, however, until it was re-introduced by Hammarsten. For the globulin precipitate, Hammarsten used the name paraglobulin.

[2] It was earlier shown by Méhu (1878) that all the serum proteins could be precipitated by saturation of serum with ammonium sulphate. According to Kauder, F. Hofmeister (in an unpublished work) was the first to use fractional precipitation with ammonium sulphate for separating proteins.

to these authors, euglobulin could also be precipitated from serum with between 0·28 and 0·33 saturated Am_2SO_4[1] while pseudoglobulin resulted from 0·34 to 0·46 saturation with Am_2SO_4.

A few years later, Freund and Joachim (1902) showed that both the euglobulin and the pseudoglobulin, precipitated with ammonium sulphate from horse serum, could be divided by dialysis into a soluble and an insoluble part. These authors therefore proposed to distinguish between the soluble euglobulin and pseudoglobulin and the water-insoluble para-euglobulin and para-pseudoglobulin. In the following year, Porges and Spiro (1903) claimed that by means of ammonium sulphate precipitation, it was possible to divide the globulin part of serum into three different fractions, each of which would yield a soluble and an insoluble fraction on dialysis.

Thence forward, the fractionation of serum with ammonium sulphate or with sodium sulphate as later advocated by Howe (1921, a), have been the methods most commonly used for preparing the serum proteins. They have also been applied to the analysis of serum with regard to its protein components[2] and much work has been expended on finding the salt concentration limits giving the most characteristic fractions.

The use of sodium sulphate as the globulin precipitant was studied by Howe, *l. c.*, who, from the shapes of the graphs where the nitrogen content of the filtrate were plotted against the concentration of sodium sulphate, came to the same conclusion as Porges and Spiro, that serum globulin could be divided into one euglobulin and two pseudoglobulin fractions. According to Howe (1925), the limits are generally put thus: euglobulin (0·30 to 0·36 saturated Am_2SO_4), pseudoglobulin I (0·36 to 0·44), pseudoglobulin II (0·44 to 0·50) and albumin (0·50 to 1·00). No reference is made to possible differences between sera from different species.

[1] Here and in the subsequent text, 0·50 saturated Am_2SO_4 means that the solution is formed by the mixing of equal volumes of a saturated ammonium sulphate solution and the solution to be precipitated. Due to the variation of the concentration of the saturated solution with temperature, it is preferable to work with a slightly diluted one. For this reason, a 4 molar ammonium sulphate solution was chosen instead of the saturated. In this case, mixtures consisting of equal volumes of 4 M-Am_2SO_4 and water (or serum) will be called, '0·50 Am_2SO_4', those of 4 parts of 4 M-Am_2SO_4 and 6 parts of water '0·40 Am_2SO_4', *etc.*

[2] In recent years the method of Butler and Montgomery (1932) (see also Butler, Blatt and Southgate, 1935), using potassium phosphate as the precipitant, has been introduced (see Wuhrmann and Wunderly, 1944).

The shape of the ammonium sulphate precipitation curve was also studied by Ruszczynski (1924) on some human and one horse sera, but no definite conclusions were reached. Another investigation of this precipitation curve, at *p*H 7 and 4·7, was carried out on human serum by Geill (1928).

Lustig (1930) made the following fractions from cow serum: euglobulin precipitated by 0·33 sat. Am_2SO_4, pseudoglobulin by 0·33 to 0·50 Am_2SO_4, albumin I by 0·54 to 0·66 Am_2SO_4, albumin II by 0·66 to 0·75 Am_2SO_4 and finally albumin III by saturating the solution with Am_2SO_4. These fractions were all dissolved in water and twice reprecipitated within the same Am_2SO_4 limits. Each of the two globulins was dissolved in water and subjected to long dialysis against water. The precipitates were then successively extracted with 0·85 per cent NaCl, 0·25 per cent Na_2CO_3 and finally with 0·02 N-NaOH. Together with the two filtrates, which contained the water-soluble parts of the two globulins, a total of eight globulin fractions was thus obtained. In the same paper Lustig showed that there was a considerable difference between the properties of the individual fractions. According to Lustig and Katz (1931) the lipids were also distributed in a characteristic way between the different globulin and albumin fractions. Similarly Lustig and Haas (1931) found that the carbohydrate contents varied greatly especially between the different subfractions.

Sørensen (1930) has fractionated both the albumin and the globulin from horse serum into a number of subfractions with varying physicochemical properties.

Hewitt (1936, 1937, 1938 a, b), using ammonium sulphate as the precipitant, has fractionated the serum proteins (generally from horse) in a special way. He has also employed his own nomenclature. In his experiments total globulin is first precipitated by 0·50 (or 0·52) sat. Am_2SO_4 and then dialysed against water at *p*H 7. The deposit formed is called euglobulin I. The filtrate is adjusted to *p*H 6 and the dialysis continued, yielding euglobulin II as a precipitate. By adding ammonium sulphate to the filtrate to 0·33 Am_2SO_4 the 'pseudoglobulin A' is obtained, whereas the 'main pseudoglobulin fraction' is precipitated by increasing the salt concentration to 0·5 Am_2SO_4. From the mother liquor from total globulin, globoglycoid, crystalbumin, and seroglycoid (perhaps also seromucoid) may be prepared after a rather complicated fractionation.

It may be a little confusing that Kendall (1937) has called the

water-soluble part of globulin obtained from human serum after 6 precipitations at 0·33 Am_2SO_4, alpha-globulin, and the corresponding pseudo-globulin, precipitated between 0·33 and 0·50 Am_2SO_4, globulin-*X*.

A very thorough investigation of the fractionation of serum and plasma has been carried out in E. J. Cohn's laboratory. As a short summary of the results obtained by Cohn *et al.* (1940, a) the Table 3, taken from their paper, may be presented.

Table 3. Serum Proteins of the Horse (Cohn *et al.*, 1940, a).

Concentration of ammonium sulphate		Type of protein precipitated	Per cent of total protein pptd.	Per cent of protein pptd.	
Moles per litre	Per cent of satn.			Water soluble	Water insoluble
1·39	0·34	Largely γ-globulin	20	71	29
1·64	0·40	γ-, β-, α-globulin	15	67	33
2·05	0·50	β-, α-globulin, mucoglobulin	14	94	6
2·57	0·62	Largely crystalline albumins	32	98	2
2·80	0·68	Crystalline albumins, haemocuprein, choline esterase, glycoprotein, phosphatase	14	99	1

Further papers from the same laboratory, dealing with the purification of the horse serum proteins, have been communicated by Green (1938), McMeekin (1939 and 1940) and Cohn *et al.* (1940, b). By dialysis and isoelectric precipitation of the total globulin, Green succeeded in preparing 3 different globulins, which she calls P_{I}, P_{II} and P_{III}.

Neurath *et al.* (1941) have prepared three different globulin fractions GI, GII and GIII in the following way: Horse serum is diluted with an equal volume of water and ammonium sulphate is added to 0·34 sat. Am_2SO_4 giving GI. GII is precipitated between 0·34 and 0·40 Am_2SO_4 and finally GIII between 0·40 and 0·53 Am_2SO_4. GI, GII and GIII are then reprecipitated several times at 0·25 to 0·34, 0·34 to 0·40 and 0·40 to 0·53 sat. Am_2SO_4 respectively. By final dialysis against water with proper adjustment of the pH, the three globulins are divided into three euglobulins PI, PII and PIII and three pseudoglobulins GI, GII and GIII. The preparation of native whole bovine albumin has been described by Putnam *et al.* (1943).

An electrophoretic study of the fractionation of horse, cow, swine,

and rabbit serum has been carried out by Svensson (1941). He showed that the alpha-globulin of Kendall and the pseudoglobulin A of Hewitt consisted of 100 per cent γ-globulin. The fraction P_I of Green and the euglobulin II of Hewitt were identical with the α-euglobulin of Tiselius. Hewitt's euglobulin I contained mainly β- and γ-globulins whereas Green's fractions P_{II} and P_{III} corresponded to γ- and β-euglobulins respectively.

2. GENERAL.

By studying under suitable conditions the sedimentation diagram of serum, we may acquire some information about the contents of albumin, globulin and a few other components in serum. By using the ultracentrifuge to follow the process of the fractionation of serum, we may hope to improve our knowledge of its protein constituents.

Before the present investigation was started, the author has always been using the classical ammonium sulphate method for preparing serum albumin and serum globulin. In this the globulin is precipitated by half saturation with ammonium sulphate and the albumin is separated from the globulin mother liquor by increasing the salt concentration until the appearance of the first permanent turbidity and the subsequent change of pH to 4·7. The first precipitates of globulin and albumin were generally re-precipitated (or in the case of horse serum albumin recrystallized) several times and finally dialysed against a suitable salt solution, or in some cases against distilled water, and eventually electrodialysed. These solutions behaved in most respects quite normally, but the author nevertheless always had the impression that in some respects their behaviour did not agree with what might be expected of a pure well-defined protein. The sedimentation diagrams were at first sight very satisfactory, but when they were examined more critically, it was observed that the peaks were not as symmetrical as might have been expected. Sedimentation equilibrium runs also gave unsatisfactory results with drifts in the values for M. In the cases of some other proteins, as for instance lactoglobulin, we were quite convinced that the results obtained were more or less definitive. These latter substances gave sedimentation equilibrium runs without drift in the values; the concentration calculated from the sedimentation diagrams, moreover, agreed with the refractometrically measured concentrations, *etc.* In other words, some irregularity seemed

to attend the examination of the serum proteins in the ultracentrifuge (see UC p. 374, or UZ p. 338, and this paper p. 16).

In 1936 the author, together with Gralén, made some centrifuge runs on some of the 'classical' fractions from horse serum, namely total globulin, pseudoglobulin, and albumin. We found that even when the albumin had been recrystallized several times it always contained small amounts (10—15 per cent) of a component with $s_{20} \sim 7$. Tiselius found that the electrophoretic diagram showed the presence of some β-globulin besides the albumin (see UC p. 374, or UZ p. 338). On the other hand globulin fractions with different solubilities and electrophoretic properties showed almost identical sedimentation diagrams (UC p. 375, or UZ p. 340).

In 1939 the author started a new investigation with the ultimate aim of finding the best method for preparing ultracentrifugally and electrophoretically (electrochemically) pure and well defined serum proteins. If this goal could eventually be reached, it was the intention to use these pure proteins in a study of the interaction of proteins in solution.

From what has been said at the beginning of this chapter, it is evident that the choise of the salt concentration limits used for the precipitation of the serum proteins is largely based upon old practice, and it may therefore be somewhat fortuitous. The purpose of the first experiments, to be described below, was to find whether it was possible for a given serum to choose the concentration range of the salt used for the fractionation in such a way that only one protein was precipitated within each range. It would therefore be valuable if the precipitation limits could be determined in a more exact manner. For practical reasons it was decided to use ammonium sulphate as the precipitant, which, on the other hand, had the disadvantage that the precipitation could not be followed by means of Kjeldahl nitrogen determinations. Several properties of the ammonium sulphate — protein mixture could be used, however, for finding the salt concentration at which the protein content of the solution changed strongly and where it varied little or not at all. The experiments, to be referred to on p. 61, showed that the precipitation limits for the different components were rather broad and that no reasonably pure components could be obtained by a single precipitation within definite limits of salt concentration.

In the next series of experiments the author explored the possibi-

lities of obtaining the pure components by submitting the different fractions to repeated further fractionations with ammonium sulphate. It was found that the preparation of the pure components in this way, if it were possible, would be a very complicated matter.

Later on a combined salt precipitation and dialysis method (partially based on the results obtained by Green (1938)) was tested. At the beginning it seemed rather promising, in so fas as fractions with distinct properties could be obtained quite easily. It was found, however, that the final purification of the fractions was quite an intricate task.

Since one of the difficulties in separating the pure globulin fractions appears to lie in the close proximity of their precipitation ranges, it would seem natural to attempt the preparation of the globulins from a serum where some of the components were known to be present in considerably smaller amounts than in the sera generally used. For this purpose a series of experiments were carried out with sera from very young calves, where it might be expected that, for instance, the γ-globulin fraction would be much smaller than in the cow serum, which had formerly been used in most cases. A very small (in some cases no) precipitate, corresponding to the γ-globulin, was also obtained, but contrary to expectation a very heavy precipitate of a new globulin was acquired. This globulin had a much lower molecular weight than hitherto observed for a serum protein, viz. about 50,000. Re-examination of the sedimentation diagrams from cow serum globulin has showed the new protein (or the *X*-protein) probably to be present in small amounts here also. For the preparation of the new protein it is undoubtedly necessary, or at least preferable, to use serum from the calf or still better from cow's fœtus.

Another line of attack was tested by first precipitating all the globulins with ammonium sulphate to 0·6 saturation, washing the precipitate with 0·6 sat. Am_2SO_4 and then successively extracting the remaining precipitate with 0·50, 0·45, 0·40, 0·35 sat. Am_2SO_4 and finally with 0·2 M-NaCl. It was hoped that the different components could be obtained in a purer condition when they were successively eluted from the larger precipitate. The author also expected that the lipid material would show greater affinity for the precipitate than for the dissolved proteins, and that the part of the precipitate which went last into solution would contain the greater part of the lipids. This

was also found to be the case, but the protein solutions obtained were just as much mixtures as those prepared in the usual way.

It is known from several investigations (*cf.* Geill. 1928 and 1938) that the precipitate obtained by addition of ammonium sulphate to serum varies considerably with the hydrogen ion concentration of the solution. So far as the author is aware, all experiments in this direction were carried out in order to determine the total amount of proteins in the different fractions, and no attention was paid to the preparative side of the problem. Some experiments were therefore made to compare the results of precipitation at different *p*H-values. These showed distinct differences between the precipitation curve at *p*H 5 and at *p*H 7. For some concentrations more protein was precipitated at the former *p*H, whereas in other cases the opposite condition prevailed. There was quite a difference, however, between the appearances of the various globulin precipitates; those obtained at *p*H 7 and below 0·45 saturation with Am_2SO_4 showed very little colour, while all the precipitates at *p*H 5 exhibited a definite yellow colour. This is probably due to a certain lability of the lipids, or at least in the binding of the pigments.

From time to time globulin fractions were prepared by dilution and acidification of serum. As the author had found earlier that special globulins present in some pathological sera could be precipitated by dialysing the sera in question, after suitable adjustment of the *p*H, against dilute phosphate buffers, it was thought worth while to test this procedure in other cases. Considerable deposits could be obtained in this way, especially when the *p*H of the dialysed serum was adjusted to about 5. These precipitates were generally strongly pigmented and sticky, probably due to their content of lipid matter. Part of them dissolved easily in 0·2 M-NaCl, but the rest was more difficult to dissolve. When the solutions were investigated in the centrifuge, several components were observed on the sedimentation diagrams. Fractionation of these euglobulin solutions showed that they contained most, if not all, of the main components present in the serum. In the preparation of the proteins from the dialysed serum less globulin was found, but it did not appear any the easier to isolate the pure components.

From all these experiments and from a study of the serum literature, the author became increasingly convinced that a large part of the trouble connected with the preparation of the serum proteins was

caused by the serum lipids. It therefore appeared to be of interest to study the influence of a removal of the lipids from the serum proteins on the properties of the latter. For this purpose different lipid removing procedures may be tried (*cf.* p. 44). These may be applied to the whole serum as well as to the different serum fractions. Another procedure is first to lyophilize the solution and then to make the extraction on the dehydrated powder. If the lipids could be taken away without damaging the proteins, it might perhaps be hoped that the main obstacle to crystallizing all the serum proteins would simultaneously be removed.

A. Choice of Salt Concentration Range for Fractionation.

In the first set of preliminary experiments, varying amounts of ammonium sulphate and water were added to 25 ml. of cow serum to give a total of 50 ml. (in a measuring flask). The content was thoroughly mixed and placed in a thermostat (at 20° C) in well-stoppered centrifuge tubes overnight. The next day the precipitate was centrifuged down sharply and the supernatant measured in a Pulfrich refractometer against a pure ammonium sulphate solution made up with exactly the same amount of salt in the same measuring flask. The difference Δn in the refractive index of the supernatant solution and the corresponding Am_2SO_4 solution was then plotted against the ammonium sulphate concentration. As long as nothing is precipitated, Δn rises slowly with increase in salt concentration, but at the same time as the precipitation starts, Δn drops and continues to do so until the salt concentration is reached where the salting out increases but slowly or not at all. This range is marked by an increase in Δn. The whole curve is thus composed of a number of rising (or horizontal) and descending lines. This method may be used to find the number of main components in a given serum and their approximate precipitation ranges. If, however, the procedure is used to determine the amount of ammonium sulphate to be employed in each step of the fractionation, the method must either be changed or the increase in the salt concentration must be produced by dialysis through suitable membranes. When dealing with larger volumes of serum the latter method seems a little too complicated and tedious. The most direct way, *i. e.* the addition of varying amount of ammonium sulphate to a constant volume of serum, was chosen.

In a later series of experiments, 5·00 ml. of serum was mixed with increasing amounts of 4 M-Am_2SO_4 (from a burette graduated in 0·02 ml.) in a number of well stoppered test tubes. The solutions were reshaken now and then and left in a thermostat overnight. The next day the precipitates were centrifuged down sharply and the supernatant solutions were subjected to the following measurements.

First the time of transit, t, between the two marks on an Ostwald viscometer, was determined for each solution, after which its refractive

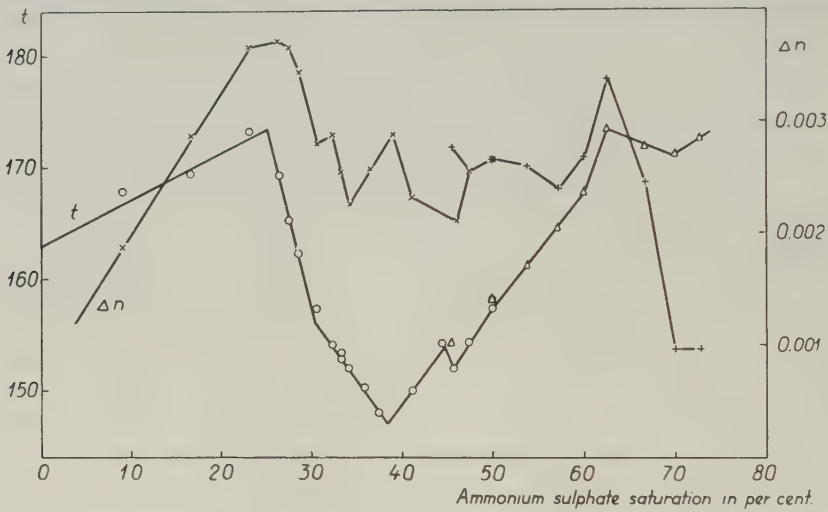

FIG. 23. The variation of viscosity and Δn with the ammonium sulphate concentration during the fractionation process (see text).

○ × and △ + refer to two different experiments with the same serum.

index was measured in a Pulfrich refractometer. In Fig. 23, t is plotted against the ammonium sulphate concentration. It is seen from the graph that as long as no precipitate is formed, t increases slightly. When the first globulin precipitates, t decreases linearly with increase in Am_2SO_4. At about '0·30 Am_2SO_4' there is a change in the slope of this line. The next change occurs at about '0·39 Am_2SO_4' where t again commences to increase with increase in salt concentration. Between '0·4' and '0·6 Am_2SO_4' the curve rises rather regularly, except perhaps around '0·45 Am_2SO_4'. Finally above '0·60 Am_2SO_4' a new change in the slope of the curve indicates the precipitation of the albumin. On the same graph, the differences between the observed and the calculated values for the refractive indices of the solutions

are also plotted. This curve shows the first part of the globulin precipitation very clearly, but the shape of the curve between 35 and 60 per cent salt seems somewhat uncertain. Finally the albumin precipitation is clearly indicated by this curve.

Fractionation Experiment No. 1. On the basis of the curve shown in Fig. 23 it was decided to take out the fractions precipitated successively at '0·31 Am_2SO_4', '0·40 Am_2SO_4', '0·50 Am_2SO_4' and '0·58 Am_2SO_4'. The filtrate from the last precipitate was acidified to about *p*H 4·7, whereby a large part of the albumin was deposited. Most of the remaining albumin came down when the concentration was finally increased to about '0·75 Am_2SO_4'. The successive precipitates[1] were named in order g_1, g_2, g_3, ga_1, a_1 and a_2.

The experiment was started with a total of 1000 ml. cow serum. The ammonium sulphate was added very slowly (10 to 30 min.) through a capillary tube ending in the middle of the solution, which was constantly and efficiently mixed by means of a stirrer. Afterwards the stirring was continued for some hours, and finally the precipitate was centrifuged tightly down. To the supernatant more ammonium sulphate was added in the same way and so on. The deposit in the centrifuge tubes was resuspended in a wash-solution, allowed to stand for a while and then centrifuged down. This procedure was carried out twice or in the case of the heavy precipitate four times. The concentration of ammonium sulphate to be used in the wash-solution was determined in accordance with the method of Sørensen and Høyrup (1917). (In this experiment the *p*H of the solution was not measured, but as nothing was done to adjust the *p*H of the ammonium sulphate solution, the *p*H of the precipitating solutions must have been of the order of 5·5—6·0.)

When the globulin is salted-out from undiluted serum, as was done in this experiment, it is quite necessary to wash the precipitate; if this is not done, too much of the albumin is carried down with the globulin. Even after such a purification, the excess protein in the deposit may be of an order of magnitude of as much as ten per cent, to judge from the work of Wiener (1911). If the serum, before the addition of ammonium sulphate, is diluted with an equal volume of water (or a weak salt solution) or still better with twice its own volume, the amount of albumin remaining in the globulin precipitate after the washing is insignificant.

The precipitates were dissolved in water or 0·2 M-NaCl to give rather concentrated protein solutions. These were filtered or more generally centrifuged clear, if they contained a small amount of insoluble material. This was generally quite insignificant and discarded. In the case of a_1 only, a part of the large precipitate was somewhat difficult to dissolve in the sodium chloride and four different extracts were made from this fraction, a small residue being discarded. Owing to an oversight the main part of the dissolved albumin from a_1 was rejected before being investigated in electrophoresis and in the centrifuge.

[1] All the separations of precipitate and filtrate described in the following account were carried out by sedimentation in Swedish angular centrifuges.

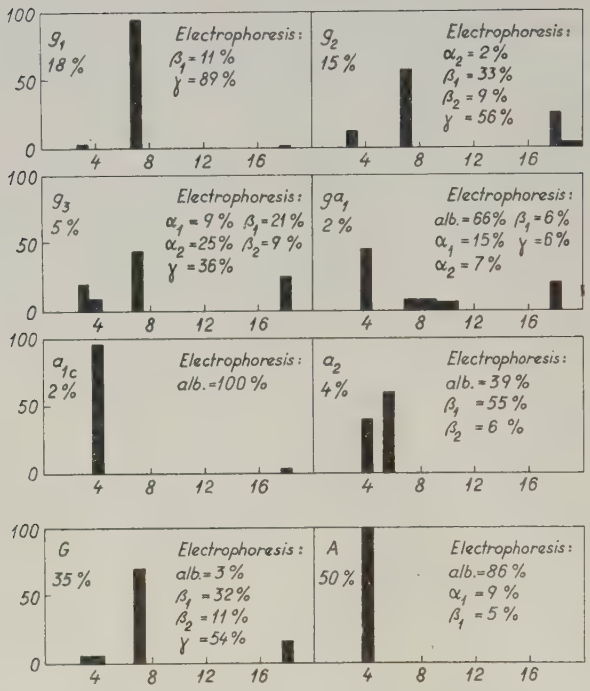

FIG. 24. Diagrammatic representation of the results obtained by ultracentrifugal examination of the different fractions from cow serum.

Upper six diagrams from Fractionation Experiment No. 1. Lower two diagrams from Fractionation Experiment No. 2. For explanation see text.

Finally, the protein solutions were dialysed first against distilled water until the main part of the ammonium sulphate had disappeared and then against frequently renewed 0·2 M-NaCl.

The different fractions were then investigated in the ultracentrifuge and by electrophoresis. For the ultracentrifuge runs the solutions were generally diluted to give $\Delta n = 200$. The results from the sedimentation experiments are schematically summarized in the upper six diagrams of Fig. 24. In the upper, left-hand corner of each diagram the name of the fraction is given together with its approximate percentage of the total protein in the serum. The heights of the rectangles give the concentrations of the different components in per cent as calculated from the sedimentation diagram. The rectangles are placed on the abscissa at values corresponding to the approximate sedimentation constants. Thus the rectangles corresponding to albumin and components with values of s_{20} of about 4 are all placed at $s_{20} = 4$.

Similarly the globulins with $s_{20} \sim 7$ are all placed at $s_{20} = 7$ and the high molecular component at $s_{20} = 18$. In cases where more inhomogeneous material was observed in the sedimentation diagram, the rectangles have been placed at the corresponding s_{20} values, but they have been broadened and their heights correspondingly reduced. Higher components than $s_{20} = 18$ have all been placed immediately above $s_{20} = 18$, irrespective of their actual sedimentation constants.

The same fractions as are given in Fig. 24 were all examined in the Tiselius electrophoresis apparatus with the long cell (see Tiselius, 1940). The solutions generally had $\Delta n = 600$ and the buffer was (0·064 M-Na_2HPO_4 + 0·008 M-NaH_2PO_4) with pH = 7·4. For the analysis, the electrophoresis diagrams were divided according to the classification of Tiselius in albumin, α_1-, α_2-, β_1-, β_2- and γ-globulin. In many cases it was very difficult to decide exactly whether a given fraction should be placed under a given component or its neighbour. The whole classification should therefore be considered as tentative and highly approximate. The results from the electrophoresis experiments are given in the upper right-hand corners of the diagrams.

Some conclusions may be drawn from Fig. 24. It is here evident that a single fractionation with ammonium sulphate yields no 100 per cent pure component. The best result in this direction was obtained with the small albumin fraction a_{1c} which was electrophoretically 100 per cent pure and ultracentrifugally 96 per cent. In the centrifuge the first globulin fraction contained only 5 per cent of components with higher and lower sedimentation constants. According to the electrophoresis diagrams, however, it should only be 89 per cent pure γ-globulin. All the other fractions were mixtures of several components.

On comparing the variations in the concentration of the different fractions from the ultracentrifuge experiments with those from electrophoresis, it is evident that the γ-globulin from electrophoresis is confined to what is ordinarily called the normal globulin component in the centrifuge (the 7-component). Similarly the electrophoretical albumin corresponds to the 'albumin' component in the centrifuge, but the 4-component in the centrifuge does not always belong to the albumin in electrophoresis (see, for instance, g_1—g_3). In this case the 4-component is probably composed of components like α_1, α_2, β_1, β_2 in the electrophoresis diagram. There seems reason to believe that the 18-component in the centrifuge corresponds to the α-component in electrophoresis, but not *vice versa*.

The difficulty in getting a proper agreement between the results obtained with the ultracentrifuge and with electrophoresis may at least to some extent be due to the fact that the various serum fractions often contain inhomogeneous heavy material. In the centrifuge this material sediments to the bottom of the cell (or rises to the top) before the measurements are started. In electrophoresis, however, the migration of all the material present is registered in the diagrams.

In some cases the wash-solution was saved for further investigation. The liquid was ultrafiltered and dialysed against saline in order to remove the ammonium sulphate and to concentrate its content of protein. Two such solutions from g_1 (the first and the fourth wash-solutions) were run in the ultracentrifuge. It proved that, as might have been expected, the first washing removed the different components in proportion to their concentrations in the mother liquor. This means that in the present case a large part of the mother liquor mechanically adhering to the precipitate has been removed. In the fourth washing, however, components already precipitated are redissolved (see Table 4).

Table 4. Percentage Composition of some Wash-Solutions from the First and Second Globulin Precipitates, according to their Ultracentrifuge Diagrams.

	4-component	7-component	20-component
First washing g_1	89	6	5
Fourth " g_1	34	61	5
First " g_2	89	9	2
Second " g_2	97	0	3

The sedimentation constants for the 'albumin' in the first washing from g_1 and in the first and second washing from g_2 correspond quite well with what would be expected for serum albumin at a similar concentration. s_{20} for the component from the fourth washing of g_1, on the other hand, shows a much lower value, which might indicate that the substance in question is not serum albumin. It may perhaps either partly consist of fetuin or of '*X*-protein' (β- or α-globulin). This is also emphasized by the electrophoresis diagrams which for g_1, g_2, and g_3 show the absence of albumin. The presence of fetuin in g_1, g_2, and g_3 is also in accordance with the precipitation range for this protein.

From this experiment, we may conclude that by a comparatively small number of washings it is possible to remove almost all the mother liquor remaining on the deposit after the precipitation and centrifugation. As has been found in another series of precipitation experiments, a similar final result may be obtained by simply diluting the serum ten times or more before adding the ammonium sulphate.

In the first set of new experiments, the serum (different from the above) was diluted with an equal volume of (0·16 M-Na_2HPO_4+0·02 M-NaH_2PO_4) and in the second set one volume of serum was diluted with four volumes of water and five volumes of (0·16 M-Na_2HPO_4+0·02 M-NaH_2PO_4). Ammonium sulphate was added to these solutions giving concentrations ranging from '0·286' to '0·444' (5 solutions) and from '0·31' to '0·50 Am_2SO_4' (6 solutions), respectively. All the first precipitates (from 0·5 c_0 serum) show amounts of the 'albumin' fluctuating between 10 per cent and 24 per cent, whereas the 'albumin' could not be detected with certainty in any of the precipitates from the most diluted serum (0·1 c_0).

We may draw the conclusion from the experiments just described, that if it is desired that the contamination with the more soluble proteins should be negligible in the precipitates, this condition may be attained by diluting the serum with an equal volume of water (or salt solution) before adding the ammonium sulphate and subsequently washing the precipitates a few times in the centrifuge tubes with a proper concentrated Am_2SO_4 solution. The same result may also be reached, if the serum is diluted ten times or more before precipitation. In this case, it is not necessary to wash the precipitate, but it suffices to centrifuge it sharply down. The first method is the most convenient for preparation, whereas the second is of value in the analytical determination of the serum proteins.

Fractionation Experiment No. 2. In the next experiment two fractions only were precipitated, both at '0·55 Am_2SO_4'.

100 ml. of cow serum was diluted with 100 ml. of (0·160 M-Na_2HPO_4+0·020 M-NaH_2PO_4) and 245 ml. 4 M-Am_2SO_4 was added as described earlier. The precipitate was washed four times with a mixture of phosphate buffer and ammonium sulphate of the same composition except that instead of serum 100 ml. of water was used. The precipitate, *G*, was dissolved in saline and ultrafiltered and dialysed against a similar solution. The mother liquor was ultrafiltered and dialysed against the following phosphate buffer (0·176 M-NaH_2PO_4+0·008 M-Na_2HPO_4) and finally concentrated to 150 ml. 15 ml. was reserved for tests and analyses by electrophoresis and ultracentrifugation. To the remainder, 165 ml. 4 M-Am_2SO_4 was added. The precipitate, *A*, and the mother liquor were treated as above.

The two fractions were thus both precipitated at the same ammonium sulphate concentration, but in the first case the *p*H of the solution was about 6·6 and the globulins were precipitated but hardly any albumin. In the second case the *p*H was about 4·6 and most of the albumin was carried down. In the lower part of Fig. 24 the results from the electrophoresis and ultracentrifuge runs on these two fractions are summarized.

The albumin fractions appear to be homogeneous in the centrifuge although it contains 9 per cent α- and 5 per cent β-globulin. The ultracentrifuge diagram from the globulin fraction corresponds quite well with what might be expected from Fractionation Experiment No. 1. The electrophoretically obtained values do not agree so well, however, with the calculated ones.

The mother liquor from the albumin precipitate was also investigated and gave an almost symmetrical 'albumin' peak in the sedimentation diagram, although the solution, according to electrophoresis, contained 13 per cent α- and 24 per cent β-globulin besides the remaining albumin.

From the first fractionation experiments it may be concluded that the precipitation ranges for the individual components overlap to a great extent. It may also be assumed that no pure protein could be prepared by a single fractionation, even if the fractions are removed between very narrow salt concentration limits. There is perhaps one exception, viz. the first globulin fraction precipitated.

In a later section examples are given of a number of different attempts to vary the conditions for the fractionation procedure.

3. FRACTIONATION OF DIFFERENT SERA.

The preliminary experiments with serum, described above, made it evident that a single fractionation with ammonium sulphate could not be expected to give a pure homogeneous protein. Since then a great number of experiments have been carried out where the conditions for the preparation of the serum proteins have been varied in several ways. Some of these investigations will now be dealt with in detail, while others will be only mentioned. Most of the fractionations, where the influences of the various experimental conditions were investigated, were carried out with cow and calf serum, but in some cases human serum was used. As the compositions of these sera are rather different, each will be treated in its own section. In addition, mention will be made of some fractionation experiments on sera from different fœtus, new-born and adult animals belonging to several species.

Nomenclature. For the sake of clarity, a special nomenclature has been used for the various fractions. To begin with, those precipitated at $\leqq 0{\cdot}50$ Am_2SO_4 are called in order g_1, g_2, *etc.*, those between $> 0{\cdot}50$ Am_2SO_4 and $\leqq 0{\cdot}60$ Am_2SO_4 ga_1, ga_2 and finally those at $0{\cdot}60$ Am_2SO_4

and $p\mathrm{H} < 5{\cdot}0$, or above 0·60 Am_2SO_4, a_1, a_2, *etc.* The euglobulins obtained by dialysis against water or very dilute salt solutions are called eug_1, eug_2, *etc.* When the 'total globulin' is used it is called *G*. In some cases fractions were obtained by successive extractions of a 'total globulin' precipitate with ammonium sulphate solutions of decreasing concentration. These extracts were called e_1, e_2, *etc.*

The fractions from a given kind of animal were all arbitrarily characterized by Greek letters in the following way:

Normal human serum, α; pathological human serum, β; serum from the umbilical cord, γ.

Cow serum, δ; calf serum, ε; fœtal serum from cow, ζ.

Sheep serum, η; lamb serum, θ (reserved); fœtal serum from sheep, ι.

Horse serum, $\varkappa$ (reserved); foal serum, λ.

Rabbit serum, μ; fœtal serum from rabbit, ν.

The number of the preparation has been given by an index to the Greek letter (several of the preparations have not been described in this account, and the corresponding indices are therefore missing). Thus $g_2\,\varepsilon_4$ means the second globulin fraction in the fourth preparation on calf serum. For human or cow serum the corresponding fractions would be named $g_2\,\alpha_4$ and $g_2\,\delta_4$. Sometimes re-fractionation was applied, and this is indicated by an addition of *a*, *b*, *c*, *etc.* to the first index number. If one of these sub-fractions was further divided, *A*, *B*, *C*, *etc.*, is added in a similar way, for instance $g_{1aA}\,\beta_4$. At a later stage, when precipitates were obtained at different *p*H values, these last are indicated by upper indices, for instance $g_1^5\,\varepsilon_{14}$, which means that this is the first globulin fraction obtained at *p*H 5. A sub-fraction of this might be $(g_1^5\,\varepsilon_{14})_2^7$.

Later on it was found preferable to adjust the lower indices to a given ammonium sulphate concentration, and the standardization was made as follows: 1, 2, 3, 4, and 5 corresponded to '0·33', '0·37', '0·40', '0·45', and '0·50 Am_2SO_4', respectively. $g_3^5\,\delta_{11}$ is thus a fraction from cow serum precipitated with '0·40 Am_2SO_4' at *p*H 5.

In most cases, the increase in the ammonium sulphate concentration was made at *p*H 7 and the fraction at *p*H 5 was deposited after the former had been removed. Sometimes, however, the first globulin fraction was obtained at *p*H 5. Not all the steps resulted in precipitates, which involves that some of the index numbers may be missing in a series of fractions.

A. Cow Serum.

a. *Adult Cow Serum.*

Some preliminary experiments with cow serum have already been mentioned in this chapter. In some of those now to be described a better separation of the individual components was attempted by means of repeated fractional precipitation at various ammonium sulphate con-

centrations, often followed by fractionation by dialysis according to Green (1938). As, however, the exact amount of protein was not determined in the different fractions or sub-fractions, I shall refrain from a more detailed description. Some of the more general results obtained may nevertheless be worth recording.

Fractionation Experiment No. 3. In one of these experiments 3 litre of serum was diluted with the same volume of water, adjusted to *p*H 6, and precipitated successively at '0·33', '0·40', and '0·50 Am_2SO_4'. The first fraction was removed for other experiments, while the two others, $g_2\,\delta_1$ and $g_3\,\delta_1$, were re-fractionated. The main part of $g_2\,\delta_1$ came down again at about '0·4 Am_2SO_4', but small deposits were also obtained at about '0·5' and '0·6 Am_2SO_4'. The latter were undoubtedly small amounts of serum albumin which had not been removed by the first washing. The second, $g_{2b}\,\delta_1$, contained three components ($s_{20} \sim 3{+}{+}$, $s_{20} \sim 7{+}{+}{+}$ and $s_{20} \sim 20{+}{+}{+}$).[1] It was first thought that the presence of the 3-component was due to contamination with albumin, but as its sedimentation constant in dilute solution was of the order of 3·5, this interpretation was somewhat dubious. Later on, when fetuin had been isolated from calf serum, the fraction was explained as belonging either to this protein or to the '*X*-protein' (α- or β-globulin).

The first fraction, $g_{2a}\,\delta_1$, was re-fractionated without investigation in the centrifuge. The precipitate at '0·33 Am_2SO_4', $g_{2aA}\,\delta_1$, showed two, perhaps three, components (3(+), 7+++++, 20+); the same was the case with the second, $g_{2aB}\,\delta_1$, at '0·40Am_2SO_4' (3(+), 7+++++, 20++); in the third, $g_{2aC}\,\delta_1$, three components were definitely present (3+, 7+++++, 20++). The two first fractions were both re-fractionated at '0·33', '0·40' and '0·50 Am_2SO_4'. The precipitates obtained were further dialysed against dilute salt solutions ($\mu = 0{\cdot}01$ to $0{\cdot}001$) at a *p*H near 6 and finally against water. The deposits from the dialysis of $g_{2aA_1}\,\delta_1$ was rather pure globulin, only a small amount of inhomogeneous material with $s_{20} \backsim 10$ being present; the supernatant was almost pure globulin with less than 1 per cent fetuin. The same result was obtained with the supernatant from the dialysis against water of $g_{2aA_2}\,\delta_1$, but the precipitates contained comparatively large amounts of the 20-component (20—30 per cent) and small amounts of fetuin (1—2 per cent). The precipitates $g_{2aA_3}\,\delta_1$ and $g_{2aB_1}\,\delta_1$ were quite insignificant in amount and were therefore discarded. The deposits from the dialysis of $g_{2aB_2}\,\delta_1$ and $g_{2aB_3}\,\delta_1$ gave results similar to those for the preceding ones, whereas the filtrates gave slightly more fetuin (< 4 per cent). These four filtrates were pooled, treated with sodium chloride to 0·2 M-NaCl and concentrated by ultrafiltration. As the properties of this solution were reminiscent of those shown by the P_{II} of Green (1938), it was called $P_{II}\,\delta_1$ and studied further by sedimentation and diffusion (see p. 159).

It was also attempted to fractionate $g_{2aA}\,\delta_1$, $g_{2aB}\,\delta_1$ and $g_{2aC}\,\delta_1$ by dialysis. This was carried out at a *p*H near 6 and the ionic strength of the outer solution was successively decreased from $\mu = 0{\cdot}02$ or $0{\cdot}01$ to $\mu = 0{\cdot}001$ and finally to $\mu \to 0$ (= water).

[1] The different numbers of + signs refer to the relative amount of the different components according to the sedimentation diagrams. + indicates that the presence of the component is just detectable and ++++++ that the great majority of the fraction consists of the component in question.

The supernatant from the last dialysis consisted mainly of globulin with $s_{20} \sim 7$ contaminated with fetuin in amounts increasing from $g_{2aA}\,\delta_1$ (< 3 per cent) to $g_{2aC}\,\delta_1$ ($\sim$ 10 per cent); in the last case some inhomogeneous material was also present. The first solution, from $g_{2aA}\,\delta_1$, appeared just as pure as that from $g_{2aA_1}\,\delta_1$. The deposits also consisted mainly of the 7-component mixed in varying proportions with the 20-component (5 to 20 per cent). After the dialysis against water, fetuin (or the '*X*-protein') was present both in the precipitate and in the supernatant.

The other globulin solution, $g_3\,\delta_1$, was also re-precipitated with ammonium sulphate and yielded the four sub-fractions $g_{3a}\,\delta_1$ to $g_{3d}\,\delta_1$. The last one consisted mainly of albumin contaminated with some inhomogeneous material. After dialysis at *p*H 6 against a phosphate mixture with $\mu = 0{\cdot}001$ the albumin appeared to be pure and the deposit proved to be a rather inhomogeneous mixture with about equal amounts of albumin and globulin.

The sedimentation diagrams from $g_{3a}\,\delta_1$ indicated the presence of at least three components (3++, 7++++, 20++). The solution had a definite blue colour, reminding of a $CuSO_4$ solution. The colour disappeared on addition of a little $Na_2S_2O_4$ but returned after a short time when air was bubbled through the solution. Similar globulin fractions from other cow sera have shown the same colour and properties. A pig globulin fraction has so far exhibited the strongest colour, being dark blue. Later on the blue substance has been concentrated by Holmberg (1944, b) in Green's (1938) P_{I}-globulin. In the same fraction he found a laccase-like enzyme, but 'the blue colour of the active globulin fraction has no importance for its catalytic function. It may possibly be due to some oxidation product with great affinity for the enzyme'.

The diagrams from $g_{3b}\,\delta_1$ recalled at first sight those from $g_{3a}\,\delta_1$, but further inspection showed that the 7-component peak was very asymmetrical (3+++, 7++++ 20++). Deposits from dialysis against phosphate at *p*H = 6 and $\mu = 0{\cdot}01$ revealed in the centrifuge the presence of four components (3+, 7+++, 8++++, 20++). Deposits from $\mu = 0{\cdot}001$ or water dialysis gave three components only (3+, 7++++, 20++ and 3+, 7+++, 20++), but the globulin peak was definitely asymmetrical in both cases. The supernatant from the water dialysis contained the same number of components, but their relative concentrations were different (3++++, 7++, 20+). There is thus a very marked difference between the water-soluble globulin solutions from the sub-fractions of $g_2\,\delta_1$ and $g_3\,\delta_1$, in the latter fetuin (or perhaps the '*X*-protein') dominates and the 20-component is present, whereas in the former the 7-component prevails and the 20-component is absent.

The result gained from these experiments is that the globulin fraction precipitated between '0·34' and '0·50 Am_2SO_4' cannot be separated into pure components by mere re-fractionation with ammonium sulphate according to the 'classical method'. By combining this with dialysis it is possible, nevertheless, to obtain a fairly pure, water soluble globulin, $P_{\text{II}}\,\delta_1$. The yield of this substance is remarkably low, however, amounting to only a very small part of the total globulin. All the other fractions were mixtures containing smaller or greater

amounts of fetuin and 20-components, besides the assemblage of proteins which is called 'normal globulin'.

Fractionation Experiment No. 4. 300 ml. of serum from a pregnant cow (in its 7th month) was mixed with an equal volume of 0·07 M-NaH_2PO_4+0·01 M-Na_2HPO_4, and globulin precipitates were removed at '0·33', '0·40', '0·45', and '0·50 Am_2SO_4' and called $g_1\,\delta_4$, $g_2\,\delta_4$, $g_3\,\delta_4$, and $g_4\,\delta_4$, respectively. Each deposit was washed twice with 100 ml. of an ammonium sulphate solution having a concentration higher by '0·02 Am_2SO_4'. The wash-solutions were added this time to the mother liquor.

$g_1\,\delta_4$ appeared homogeneous in the centrifuge, but it must have contained both fetuin and the 20-component in small amounts besides the 7-component, since after dialysis some of the deposits contained 2 per cent of the 20-component, whereas the last supernatant included 1—2 per cent fetuin. $g_1\,\delta_4$ was re-precipitated at the following concentrations '0·25', '0·30', '0·35', '0·40', and '0·45 Am_2SO_4'. The deposits were washed with 25 ml. of a solution with a concentration higher by '0·05 Am_2SO_4'. The wash solution from $g_{1a}\,\delta_4$ contained a considerable amount of protein, that from $g_{1b}\,\delta_4$ somewhat less, those from $g_{1c}\,\delta_4$ and $g_{1d}\,\delta_4$ insignificant amounts and that from $g_{1e}\,\delta_4$ none. Ultracentrifugal inspection of $g_{1a}\,\delta_4$ showed that it consisted mainly of the 7-component, but besides this some inhomogeneous material was present. This was also quite evident from a diffusion experiment on the same material, where $D_A = 2{\cdot}68$ and $D_m = 3{\cdot}27$. This difference between the two values would undoubtedly have been considerably reduced, if the solution had been spun in the air-driven centrifuge before the diffusion experiment (*cf.* p. 160 ff.).

The fractions $g_{1b}\,\delta_4$, $g_{1c}\,\delta_4$ and $g_{1d}\,\delta_4$ showed properties similar to those of the original $g_1\,\delta_4$. $g_{1e}\,\delta_4$ amounted to very little and was discarded without further investigation.

The appearance and the 'mechanical' consistency of the precipitates after they had been centrifuged down, were very characteristic. $g_1\,\delta_4$ was tough and took some time to dissolve. $g_2\,\delta_4$ was less tough and went more rapidly into solution. The following fractions gave loose precipitates. $g_{1a}\,\delta_4$ was gritty, stif and slow in dissolving. $g_{1b}\,\delta_4$ was tough and viscous. $g_{1c}\,\delta_4$ appeared rather compact, but was comparatively easy to dissolve. $g_{1d}\,\delta_4$ was loose and went rapidly into solution.

$g_2\,\delta_4$ and its sub-fractions at '0·30', '0·35', '0·40', and '0·45 Am_2SO_4' gave very much the same results as found for the sub-fraction of $g_{2a}\,\delta_1$ in the preceding experiments, whereas $g_3\,\delta_4$ and $g_4\,\delta_4$ recalled the sub-fractions from $g_3\,\delta_1$.

The water-soluble globulin fractions from $g_{1b}\,\delta_4$, $g_{1c}\,\delta_4$ and $g_{2b}\,\delta_4$ were pooled, tested according to Green (1938), and found to conform to her P_{II}; the pooled solution was called $P_{II}\,\delta_4$. It was divided into two fractions $P_{II,1}\,\delta_4$ and $P_{II,2}\,\delta_4$ by precipitation at '0·33' and '0·50 Am_2SO_4'. Both were dialysed against dilute salt solutions and finally against water. $P_{II,1}\,\delta_4$ was practically pure globulin according to the sedimentation diagrams, while $P_{II,2}\,\delta_4$ contained some fetuin. $P_{II,1}\,\delta_4$ was also investigated in electrophoresis at *p*H 4·6 and showed a single but asymmetrical peak with a huge reversible boundary spreading. It should undoubtedly be classified as an assemblage of γ-globulins according to the term employed by Kekwick and McFarlane (1943, p. 100).

The mother liquor from $g_4\delta_4$ was precipitated at '0·55' and '0·60 Am_2SO_4' and the *p*H changed to 5·1. Finally a total of eight different albumin fractions were taken at different *p*H values and ammonium sulphate concentrations.

The solutions $ga_1\delta_4$, $ga_2\delta_4$ and $a_1\delta_4$ were each transferred to *p*H 7·0 and precipitated by half-saturation with ammonium sulphate. The deposits were dissolved in water, pooled and precipitated again at *p*H 7·0 and '0·50 Am_2SO_4'. The procedure was repeated and the resulting solution was called $gg_2\delta_4$, as it should correspond to the globoglycoid of Hewitt (1938, a, see also Svensson, 1941). The sedimentation diagrams from $gg_2\delta_4$ showed two main components, the larger with a value of s_{20} between those for fetuin and albumin, and the other corresponding in this respect to the globulin. By dialysis against weak salt solutions at *p*H 6 the relative amount of the 7-component increased in the deposits while it decreased in the supernatant. The experiments did not appear very promising for the preparation of a pure component, and they were dropped for the time being. It is of interest to note that Svensson found both α- and β-globulin together with small amounts of γ-globulin in his globoglycoid preparations.

The last albumin fraction, $a_8\delta_4$, was examined both in the centrifuge and in electrophoresis and each experiment showed the presence of two components.

The result of this experiment confirms the conclusions made at the end of the preceding experiment. It was also found that the globulin first precipitated at '0·33 Am_2SO_4' was comparatively pure according to the sedimentation diagram, and the same was the case with its sub-fractions obtained by re-precipitation with ammonium sulphate. It seems necessary, however, to be lavish with the material, if comparatively pure globulin fractions are to be obtained. In an attempt to find methods more economical with the material, new experiments were carried out with serum from the calf (see later) and the cow. In these investigations the classical fractionation technique was varied in different ways.

Fractionation Experiment No. 5. In this case, one litre of serum from a twelve year old cow was dialysed against 0·01 M–NaH_2PO_4 in the cold room for two days. Two deposits were removed during this period without being further investigated. A third precipitate, $eug_3\delta_{7,8}$, was formed, when the dialysed solution was diluted with an equal volume of water.

The supernatant was divided into two aliquots, one of which was kept at *p*H 5·0 (δ_7) and the other transferred to *p*H 7 (δ_8). The two solutions were successively fractionated[1] with 'Am_2SO_4, *p*H 5' and 'Am_2SO_4, *p*H 7', respectively, at the following concentrations '0·33', '0·40', '0·45', '0·50', '0·55', and '0·60 Am_2SO_4'. The mother liquor from $ga_1\delta_8$ did not produce any precipitate when the salt concentration was increased to '0·60 Am_2SO_4'. The *p*H of the solution was then changed from 7 to 5·05 and $ga_2\delta_8$ was obtained. The supernatant, however, on standing successively developed three more deposits, although the *p*H of the solution did not change more than from 5·05 to 5·02. Finally three different albumin fractions were obtained.

The precipitates were washed twice with 100 ml. of solutions more concentrated by '0·03 Am_2SO_4' than the mother liquor. The loss of material in the wash-solutions

[1] A detailed description of a fractionation experiment has been given on p. 85 ff.

TABLE 5. COMPOSITION OF DIFFERENT FRACTIONS OBTAINED FROM COW SERUM AT *p*H 5.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
eug$_3$ $\delta_{7,8}$	0·90	585					4·42	36	6	0·06	6·72	213	36	0·32	16·9	157	27	0·24	64	11	0·10	20	0·18	0·18
g$_1$ δ_7	1·73	631									7·02	410	65	1·13					160	25	0·43	10	0·17	0·35
g$_2$ δ_7	5·42	516	*3·6*	8	2	0·11					6·67	308	60	3·25	15·0	52	10	0·54	89	17	0·92	11	0·60	1·08
g$_3$ δ_7	2·06	558	*3·54*	40	7	0·15					6·52	269	48	0·99	15·5	38	7	0·14	84	15	0·31	23	0·47	0·41
g$_4$ δ_7	0·66	491					*3·75*	81	16	0·11	6·71	199	41	0·27					71	14	0·09	29	0·19	0·13
ga$_1$ δ_7	0·56	515					*3·58*	109	21	0·12	6·57	132	26	0·14					95	18	0·10	35	0·20	0·11
ga$_2$ δ_7	2·06	541					4·02	390	72	1·48												28	0·58	0·41
a$_1$ δ_7	2·51																							0·50
a$_2$ δ_7	0·53																							0·11
a$_3$ δ_7	0·68																							0·14
a$_4$ δ_7	0·05																							0·01
'Total globulin'[1]	10·77				2	0·26			2	0·17			55	5·96			9	0·92		17	1·85	15	1·61	2·15

[1] 'Total globulin' except *eug*$_1$ and *eug*$_2$.

TABLE 6. COMPOSITION OF DIFFERENT FRACTIONS OBTAINED FROM COW SERUM AT *p*H 7.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			Δn	in		not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.		%	g.	%	g.	
eug_3 $\delta_{7,8}$	0·90	585					4·42	36	6	0·06	6·72	213	36	0·32	16·9	157	27	0·24	64	11	0·10	20	0·18	0·18
g_1 δ_8	3·87												[1]62	2·40						[1]24	0·93	[1]14	0·54	0·77
g_2 δ_8	2·90	519	*3·7*	14	3	0·09					6·48	334	64	1·85	16·7	17	3	0·09	19	4	0·12	26	0·75	0·58
g_3 δ_8	1·65	564	*3·38*	58	10	0·17					6·61	158	28	0·46	15·6	152	27	0·45	107	19	0·31	16	0·26	0·33
g_4 δ_8	0·38	491	*3·45*	105	21	0·08					6·59	105	21	0·08	16·9	82	17	0·06				41	0·16	0·08
ga_1 δ_8	0·37	381					*3·77*	120	32	0·12	6·45	47	12	0·04	18·4	14	4	0·02				52	0·19	0·07
ga_2 δ_8	0·50	350					*3·82*	95	27	0·14									159	46	0·23	27	0·13	0·10
ga_3 δ_8	0·04																							0·01
ga_4 δ_8	0·70																							0·14
ga_5 δ_8	1·20																							0·24
a_1 δ_8	1·34																							0·27
a_1' δ_8	0·54																							0·11
a_2 δ_8	0·71	420					4·32	58	14	0·10									22	5	0·04	81	0·57	0·14
a_3 δ_8	0·65	365					*3·85*	103	28	0·18									39	11	0·07	61	0·40	0·13
'Total globulin'[2]	9·70				3	0·34			1	0·06			53	5·11			9	0·84		15	1·46	19	1·89	1·94

[1] Mean values from several experiments.

[2] 'Total globulin' except eug_1 and eug_2.

was determined for some of the fractions. This loss varied considerably, but was in most cases of the order of magnitude of 5 per cent, when referred to the washed precipitate. In some of the fractions, which were difficult to wash, as for instance the *ga*'s, the loss sometimes amounted to as much as 20 per cent. In a few cases it was only 1 per cent. The results from the ultracentrifugal examination of some of the fractions have been summarized in Tables 5 and 6.

In this and the following tables the column headed: 'Grams of protein' gives the total amount of protein in the fraction as calculated from the total volume of the fraction and its concentration. The latter has been calculated from the Δn of the solution, generally after dialysis against 0·2 M-NaCl, assuming that $\Delta n = 200$ is equal to a concentration of 1 g. protein/100 ml. solution. As, however, the specific refraction increment, dn/dc, for most of the serum proteins is of the order 0·00180 to 0·00200 ($180 < \Delta n$/g. protein per 100 ml. of solution < 200), the amounts of protein for the individual fractions given in the tables are minimum values. The column 'Δn in run' refers to the Δn of the centrifuged solution. In the columns headed 'Fetuin', 'Albumin', *etc.*, s_{20} is the sedimentation constant as measured from the sedimentation diagrams; sometimes s_{20} for a given component has appeared too high to be placed under 'Fetuin' and too low for the 'Albumin'. It has then tentatively been placed under one of them and the figure for s_{20} has been printed in italic. The 'component' in question could be a mixture of fetuin and albumin, or of either of these and the '*X*-protein'; it could perhaps also consist of the '*X*-protein' alone. The average value in 'Δn' is calculated from the areas of the corresponding peaks in the diagrams (UC p. 293 ff. or UZ p. 263 ff.). For each component, $\frac{\Delta n \cdot 100}{\Delta n \text{ in run}}$ is given under '%'. Finally the amounts of the different components present in each fraction, 'g.', are computed from this percentage and 'Grams of protein'. In many cases the solutions contained inhomogeneous material, part of which was visible in the diagrams and could be calculated from the curves. This has been placed under the heading: 'Inhomogen. material in sedimentation diagram'. The '%' in the next column 'Inhomogen. material not in sedimentation diagram' is calculated from the difference between 'Δn in run' and the sum of the Δn's for the different components plus 'Inhomogen. material in sedimentation diagram'. It thus includes the inhomogeneous material sedimenting to the bottom (or occasionally to the top) of the centrifuge cell before the measurements were started, as well as the material that is not accounted for owing to the presence of the paraffin oil (see p. 17). As, however, the values in this column are derived as differences, they may be encumbered with comparatively large errors.

It should be remarked that the amounts of protein given for the individual components in the various fractions are minimum values. Quite probably part of the amount included in 'Inhomogen. material not in sedimentation diagram' would have increased the values for the different

components by perhaps 10 per cent in the case of the globulins and somewhat more for the other components, if the centrifuged solution had not been covered with paraffin oil. It may also be emphasized that the values given for the individual fractions are not identical with their actual amounts in the original serum. The first globulin fraction will be almost correct except for small unavoidable losses in the different manipulations. Not insignificant quantities of substances belonging to the later fractions are discarded together with the various wash-solutions. This implies that the larger the globulin fractions are, the more albumin must have disappeared with the wash-solutions before being properly precipitated. The amounts of protein in the different fractions given in the tables thus represent the yields in actual preparations and are not identical with an analysis of the original serum.

It is seen from the tables that more globulin is brought down in the first fraction at *p*H 7 (δ_8) than in that at *p*H 5 (δ_7). The precipitation of the 'albumin' (*ga*) fraction starts at a lower salt concentration in δ_7 than in δ_8, and the separation of the 'albumin' and the globulin fraction is thus not as good at *p*H 5 as at *p*H 7. On the other hand, the precipitation of the 20-component is accomplished at a lower salt concentration at *p*H 5 than at *p*H 7. In the next experiment it was investigated whether some advantage could be gained by not keeping the *p*H of the solution constant during the whole fractionation, but taking some of the precipitates at *p*H 5 and others at *p*H 7.

Fractionation Experiment No. 6. A part of the serum used in the preceding experiment was dialysed, after adjustment of the *p*H to 6, against a phosphate buffer with *p*H about 6 and $\mu = 0{\cdot}01$. The resulting deposit, $eug_1\,\delta_9$, was washed with 100 ml. of a similar phosphate buffer. The *p*H of the dialysate was then changed to 5 and the solution was subjected to dialysis against 0·01 M-NaH_2PO_4. The resulting precipitate, $eug_2\,\delta_9$, was washed twice with 100 ml. 0·01 M-NaH_2PO_4. The supernatant, which had *p*H = 4·48, was diluted with an equal volume of water. This gave rise to a new deposit, $eug_3\,\delta_9$, which was washed with 100 ml. of 0·005 M-NaH_2PO_4.

The *p*H of the dialysed and diluted serum was then adjusted to 5·0 and the globulin fractions were alternately taken at *p*H 5 and 7 successively at the following concentrations '0·33', '0·40', '0·45', and '0·50 Am_2SO_4' (no precipitate at '0·50, *p*H 5') in the order given in Table 7. The *ga* fractions were taken at '0·55, *p*H 7', '0·55, *p*H 5' (no precipitate), '0·60, *p*H 7' and '0·60 Am_2SO_4, *p*H 5·1'. The albumin fractions came down at '0·60, *p*H 4·9', '0·60, *p*H 4·7', '0·60, *p*H 4·0'. $a_4\,\delta_9$ was finally precipitated after saturation with ammonium sulphate.

The heavy precipitates were washed twice with 100 ml. of solutions more concentrated by '0·03 Am_2SO_4' having the proper *p*H, whereas only 50 ml. was used for the smaller precipitates. There was one exception, however, since $g_1^5\,\delta_9$ was washed with 100 ml. of '0·35 Am_2SO_4, *p*H 5'. The amounts of protein in the wash solutions from $g_1^5\,\delta_9$, $g_1^7\,\delta_9$, and $g_2^7\,\delta_9$ were found to be 15, 6 and 3 per cent, respectively, of

TABLE 7. COMPOSITION OF DIFFERENT FRACTIONS FROM COW SERUM (FRACTIONATION EXPERIMENT NO. 6).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$eug_1\ \delta_9$	0·46	138					*3·7*	8	5	0·02	6·91	118	76	0·35	17·9	30	19	0·09						0·09
$eug_2\ \delta_9$	0·14	84					*4·0*	10	12	0·02	7·07	16	19	0·03	22·3	12	14	0·02	6	7	0·01	48	0·06	0·03
$eug_3\ \delta_9$	0·34	271									7·17	7	3	0·01								97	0·33	0·07
$g_1^5\ \delta_9$	3·16	617									7·02	141	23	0·73					33	5	0·16	72	2·27	0·63
$g_1^7\ \delta_9$	3·71	603									6·79	358	59	2·19					162	27	1·00	14	0·52	0·74
$g_2^7\ \delta_9$	3·61	597									6·68	309	52	1·88	16·3	30	5	0·18	146	24	0·87	19	0·68	0·72
$g_2^5\ \delta_9$	0·26	591	3·4	41	7	0·02					6·39	46	8	0·02	17·3	242	41	0·11	26	4	0·01	40	0·10	0·05
$g_3^7\ \delta_9$	0·88	555	*3·7*	16	3	0·03					6·69	223	41	0·36	16·0	72	13	0·11	51	9	0·08	34	0·30	0·18
$g_3^5\ \delta_9$	0·33	552	3·22	62	11	0·04					6·69	119	22	0·07	17·0	129	23	0·08				44	0·14	0·07
$g_4^7\ \delta_9$	0·56	598	*3·56*	94	16	0·09					6·37	227	38	0·21	17·8	46	8	0·05				38	0·21	0·11
$ga_1^7\ \delta_9$	0·41	635					*3·49*	133	21	0·09	6·47	266	42	0·17					218	34	0·14	3	0·01	0·08
$ga_2^7\ \delta_9$	0·08	255					*3·98*	77	30	0·02									119	47	0·04	23	0·02	0·02
$ga_2^5\ \delta_9$	0·16																							0·03
$a_1\ \delta_9$	2·63																							0·53
$a_2\ \delta_9$	2·25																							0·45
$a_3\ \delta_9$	0·86	491					4·28	324	66	0·57									113	23	0·20	11	0·09	0·17
$a_4\ \delta_9$	1·11	428					*3·78*	160	37	0·41									60	14	0·16	49	0·54	0·22
'Total globulin'	13·45				1	0·18				0·04			44	5·85			5	0·64		16	2·13	34	4·61	2·69

TABLE 8. COMPOSITION OF VARIOUS OF THE ABOVE FRACTIONS AFTER REFRACTIONATION WITH AMMONIUM SULPHATE.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$(g_1^5\ \delta_9)_1^5$	0·21	373									7·03	227	61	0·128					110	29	0·061	10	0·021	
$(g_1^5\ \delta_9)_1^7$	0·03	154									7·27	86	56	0·017					34	22	0·006	22	0·007	
$(g_1^5\ \delta_9)_2^7$	0·05	209	*3·69*	11	5	0·002					7·13	141	68	0·034					33	16	0·008	11	0·006	
$(g_1^5\ \delta_9)_3^7$	0·05	115	*2·6*				4·5				6·6													Medley
$(g_2^7\ \delta_9)_1^7$	1·15	549									6·95	264	48	0·552	20·9	42	8	0·092	226	41	0·472	3	0·034	
$(g_2^7\ \delta_9)_2^7$	0·43	643									6·59	366	57	0·245	15·8	34	5	0·022				38	0·163	
$(g_2^7\ \delta_9)_3^7$	0·29	579									6·72	234	40	0·116	15·6	143	25	0·073	53	9	0·026	26	0·075	
$(g_2^7\ \delta_9)_3^5$	0·12	491	3·33	26	5	0·006					7·04	108	22	0·026	18·4	75	15	0·018				58	0·070	
$(g_2^7\ \delta_9)_a$	0·08	302					*3·98*	29	10	0·008	6·90	149	49	0·039					41	14	0·011	27	0·022	
$a_{4a}\ \delta_9$	0·18	178					*3·97*	38	21	0·038									12	7	0·012	72	0·130	
$a_{4b}\ \delta_9$	0·35	373					*3·68*	233	63	0·220									94	25	0·088	12	0·042	

the fraction in question. This indicates that a difference of '0·02 Am_2SO_4' between the precipitation value and the wash-solution is too small, but that '0·03' seems to be reasonable.

The results from the ultracentrifugal examination of most of the fractions have been given in Table 7.

It is seen from the table that the first euglobulin fraction contains a considerable amount of globulin, whereas the second, and especially

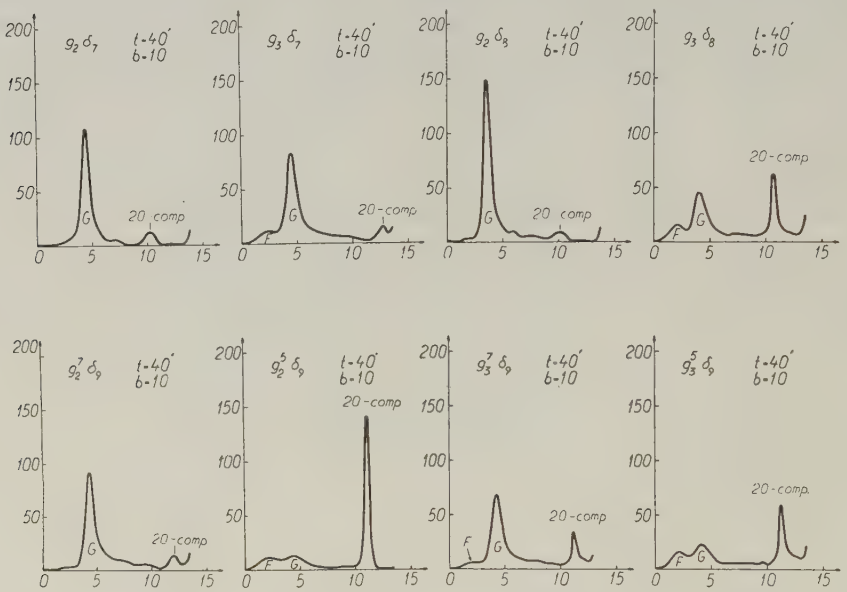

Fig. 25. Sedimentation diagrams for the globulin fractions from cow serum precipitated at '0·40' and '0·45 Am_2SO_4'.

All diagrams have been reduced to the same protein concentration ($\Delta n = 200$).

The precipitations have been made at *p*H 5 for $g_2\,\delta_7$ and $g_3\,\delta_7$, at *p*H 7 for $g_2\,\delta_8$ and $g_3\,\delta_8$, and alternately at *p*H 7 and 5 for the four fractions from δ_9.

the third, euglobulin consists to a large extent of inhomogeneous material. The same is the case with $g_1^5\,\delta_9$.

If Tables 5, 6 and 7 are compared it seems likely that the large quantity of inhomogeneous material in $g_1^5\,\delta_9$ might just as well have been precipitated in the *eug*-fractions.

From a comparison of the fractions from '0·40' and '0·45 Am_2SO_4' of δ_7, δ_8 and δ_9, it is evident that the precipitation with the *p*H alternating between *p*H 5 and 7 seems to offer certain advantages in the separation of the 20-component. The sedimentation diagrams from corresponding runs in the ultracentrifuge are given in Fig. 25.

It was attempted in different ways to re-fractionate some of the above fractions. Thus $g_1^5\,\delta_9$ was dialysed first at *p*H 7 against a phosphate buffer with $\mu = 0{\cdot}01$, but no precipitation occurred. When the *p*H was changed to 5·0, 60 to 70 per cent of the dissolved material came down as a heavy deposit. This was extracted first with 0·2 M-NaCl and then with 0·2 M-Na_2HPO_4, but comparatively little went into solution (about one sixth of the whole). It was found to consist of globulin mixed with inhomogeneous material having both higher and lower sedimentation constants than the globulin. The remainder of the precipitate could be brought into solution by treatment with 1 M-NaCl or with 0·2 M-Na_2HPO_4 at 40°C overnight. Ultracentrifugal examination of the two solutions, after dialysis against 0·2 M-NaCl, showed that each contained a wide variety of materials. This agrees well with the large amount of inhomogeneous material found in the original $g_1^5\,\delta_9$ solution (see Table 7). An inspection of the filtrate from the dialysis of $g_1^5\,\delta_9$ showed that the solution contained 53 per cent globulin and 47 per cent inhomogeneous material, which indicates some improvement when compared with the values given in Table 7.

Attempts were made also to purify $g_2^7\,\delta_9$ by re-precipitation with ammonium sulphate, but in vain. Only the relative concentration of the 20-component was considerably increased in two of the fractions (see Table 8).

In the same table, the values for two sub-fractions from $a_4\,\delta_9$ are given. They were prepared in the following way: $a_4\,\delta_9$ was dialysed against water, and adjusted to *p*H 5. The precipitate formed amounted to about one fifth of the total and was discarded. To the supernatant, ammonium sulphate (*p*H 5) was added first to '0·50', (no precipitate) and then to '0·60 Am_2SO_4', which gave $a_{4a}\,\delta_9$. Change of the *p*H of the mother liquor to 4·7 and 4·1 did not produce any deposit. After saturation with ammonium sulphate $a_{4b}\,\delta_9$ was obtained. It is seen from Table 8 that $a_{4b}\,\delta_9$ contains less inhomogeneous heavy material than $a_4\,\delta_9$, but the proportion between 'albumin' and 'inhomogeneous material in sedimentation diagram' is practically the same. The fractionation has thus resulted in the removal of the larger part of the heavy, inhomogeneous material (*cf.* $a_{4a}\,\delta_9$ in Table 8).

Fractionation Experiment No. 7. In an experiment with another serum, this was adjusted to *p*H 4·5 and dialysed against water. The first heavy precipitate from 500 ml. of serum was washed twice with 100 ml. of water. In this way 1·4 g. of substance was rejected with the wash-solution. An extraction with 30 ml. of 0·2 M-NaCl brought only 0·12 g. $eug_1\,\delta_{10}$ into solution, while the remainder dissolved in 70 ml. 0·2 M-Na_2HPO_4 ($eug_1'\,\delta_{10} = 1{\cdot}11$ g.). Ultracentrifugal examination showed that $eug_1\,\delta_{10}$ consisted mainly of albumin (62 per cent), 29 per cent of inhomogeneous material and 9 per cent 20-component, whereas $eug_1'\,\delta_{10}$ contained 77 per cent inhomogeneous material, 9 per cent albumin and 14 per cent globulin. The rest of the experiment gave results similar to those described below.

Fractionation Experiment No. 8. Five hundred ml. of the same serum was adjusted to *p*H 5·50 and dialysed against water. The precipitate, $eug_1\,\delta_{11}$, was washed twice with 100 ml. of water. The wash-solution, which contained 0·75 g. of nitrogeneous material, was discarded. The *p*H of the supernatant was changed back to 5·50 from 5·30, and after further dialysis $eug_2\,\delta_{11}$ was formed and treated as above. The supernatant (*p*H = 5·49) was diluted with water to a total of 1500 ml., whereby $eug_3\,\delta_{11}$ was produced.

TABLE 9. COMPOSITION OF DIFFERENT FRACTIONS FROM COW SERUM (FRACTIONATION EXPERIMENT NO. 8).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$eug_1\ \delta_{11}$	2·08	595					*4·00*	47	8	0·17	6·7[1]	299	50	1·04	12·8	67	11	0·23				31	0·64	0·42
$eug_1'\ \delta_{11}$	0·51	341					4·58	32	9	0·05	6·04	130	38	0·19	13·7	36	11	0·06	26	8	0·04	34	0·17	0·10
$eug_2\ \delta_{11}$	0·46																							0·09
$eug_3\ \delta_{11}$	0·71																							0·14
$g_1^5\ \delta_{11}$	0·61	611									6·56	364	60	0·36	18·0	5	1	0·01				39	0·24	0·12
$g_1^7\ \delta_{11}$	1·93	625					*3·66*	6	1	0·02	5·91	438	70	1·35								29	0·56	0·39
$g_2^7\ \delta_{11}$	2·31	644									6·24	370	58	1·34	14·2	92	14	0·32				28	0·65	0·46
$g_3^5\ \delta_{11}$	1·81	618					*3·61*	76	12	0·22	6·89	101	16	0·29	*24·8*	24	4	0·07	41	7	0·13	61	1·10	0·36
$ga_1^7\ \delta_{11}$	0·31	349					*3·70*	114	33	0·10	6·94	88	25	0·08								42	0·13	0·06
$ga_1^5\ \delta_{11}$	7·48	787					3·92	260	33	2·47									119	15	1·12	52	3·89	1·50
$ga_2^5\ \delta_{11}$	3·68	630					4·19	480	76	2·80												24	0·88	0·74
$a_2\ \delta_{11}$	0·35																							0·07
$a_3\ \delta_{11}$	0·09	108					*3·32*	37	34	0·03									19	18	0·01	18	0·02	0·02
							4·45	32	30	0·03														
'Total globulin'[2]	9·25								5	0·46			49	4·57			8	0·69		2	0·17	36	3·36	1·85

[1] Asymmetric peak.

[2] Except $eug_2\ \delta_{11}$ and $eug_3\ \delta_{11}$.

TABLE 10. COMPOSITION OF DIFFERENT FRACTIONS FROM $eug_1''\ \delta_{11}$ AFTER RE-PRECIPITATIONS WITH AMMONIUM SULPHATE.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material					Grams of protein per 100 ml. of serum
																			in sedimentation diagram			not in sedimentation diagram		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$eug_1''\ \delta_{11}$	1·74	500					*3·84*	48	9	0·16	6·91	158	32	0·55	16·2	59	12	0·21				47	0·82	
$(eug_1''\ \delta_{11})_1^5$	0·27	216					*3·97*	9	4	0·011	7·37	36	17	0·046	20·4	10	5	0·014	26	12	0·032	62	0·167	
$(eug_1''\ \delta_{11})_2^5$	0·46	380					*3·64*	26	7	0·032	7·27	140	37	0·170	18·5	91	24	0·111	85	22	0·101	10	0·046	
$(eug_1''\ \delta_{11})_3^7$	0·48	604					4·35	27	5	0·024	6·66	238	39	0·187	15·8	114	19	0·091	49	8	0·039	29	0·139	
$(eug_1''\ \delta_{11})_3^5$	0·11	271					*4·05*	17	6	0·007	7·03	71	26	0·029	20·1	66	25	0·027	11	4	0·004	39	0·043	
$(eug_1''\ \delta_{11})_4^5$	0·17																							
$(eug_1''\ \delta_{11})_n$	0·11	185					4·23	44	24	0·026									104	56	0·062	20	0·022	

The *p*H of the supernatant was changed to 5·0 and solid ammonium sulphate was added to make the solution '0·35 Am_2SO_4'. The amount of the salt to be used was determined by means of a diagram based on that given by Quensel (1942, p. 30). The deposit, $g_1^5\,\delta_{11}$, was washed twice with 100 ml. '0·40 Am_2SO_4, *p*H 5'. The following precipitates were obtained in order: at '0·35 Am_2SO_4, *p*H 7', $g_1^7\,\delta_{11}$; at '0·40 Am_2SO_4, *p*H 7', $g_2^7\,\delta_{11}$; at '0·40 Am_2SO_4, *p*H 5', no precipitate, but the solution slightly turbid; at '0·50 Am_2SO_4, *p*H 5', $g_3^5\,\delta_{11}$; at '0·50 Am_2SO_4, *p*H 7', no precipitate; at '0·55 Am_2SO_4, *p*H 7', $ga_1^7\,\delta_{11}$; at '0·55 Am_2SO_4, *p*H 5', a very heavy precipitate (probably mainly albumin) $ga_1^5\,\delta_{11}$; at '0·60 Am_2SO_4, *p*H 5', a heavy precipitate $ga_2^5\,\delta_{11}$. The mother liquor was then acidified first to *p*H 4·70 (no precipitate) and afterwards to *p*H 4·1, where $a_2\,\delta_{11}$ came down. On saturation with ammonium sulphate $a_3\,\delta_{11}$ was finally obtained. The washings were carried out as described for $g_1^5\,\delta_{11}$ with solutions having a '0·05 Am_2SO_4' higher salt concentration and the proper *p*H.

The result of an ultracentrifugal examination of most of the fractions is given in Table 9. The precipitate $eug_1\,\delta_{11}$ was extracted first with 70 ml. 0·2 M-NaCl, which yielded $eug_1\,\delta_{11}$, and then with 0·2 M-Na_2HPO_4, which gave $eug_1'\,\delta_{11}$.

It is seen from Table 9 that the g_1-fractions from δ_{11} are somewhat purer in globulin than the corresponding ones from δ_9. On the other hand, much more globulin is 'lost' in the *eug*-fractions from δ_{11} than in those from δ_9.

A purification of $g\,a_1^5\,\delta_{11}$ was attempted but without much success. A large part of the inhomogeneous heavy material could be removed from the solution at '0·55 Am_2SO_4, *p*H 7', but when the main part of the protein was re-precipitated at '0·55 Am_2SO_4, *p*H 5', it was nevertheless found to show considerable amounts of inhomogeneous material in the sedimentation diagram.

It was also investigated whether it was possible to purify the globulin from the *eug*-fraction. The $eug_1\,\delta_{11}$ was to be used for this purpose, but it was found to contain some deposit corresponding to about one sixth of the original substance. The supernatant, $eug_1''\,\delta_{11}$, was subjected to fractionation with ammonium sulphate as follows: At '0·29 Am_2SO_4, *p*H 5' $(eug_1''\,\delta_{11})_1^5$ was obtained; at '0·33 Am_2SO_4, *p*H 5', $(eug_1''\,\delta_{11})_2^5$; at '0·40 Am_2SO_4, *p*H 7', $(eug_1''\,\delta_{11})_3^7$; at '0·40 Am_2SO_4, *p*H 5', $(eug_1''\,\delta_{11})_3^5$; at '0·45 Am_2SO_4, *p*H 5', $(eug_1''\,\delta_{11})_4^5$. The final precipitate did not occur until the solution was saturated with ammonium sulphate.

It is seen from Table 10 that hardly any purification of the globulin was produced by the re-precipitation with ammonium sulphate of the *eug*-fraction. Even the small fraction with $s_{20} \sim 4$, probably the '*X*-protein' (α- or β-globulin), was present in all the precipitates.

From the experiments in this section, it may be concluded that the euglobulin, formed by the dialysis of serum against water at a *p*H around 5, is quite unsuitable as a 'raw material' for the preparation of a pure globulin fraction.

It may be inferred from the results given in Table 7 that the use of 'alternating *p*H' during the fractionation process offers definite advantages in the preparation of the serum globulins.

b. Calf Serum.

The fractionation experiments with cow serum had shown the great difficulties encountered in attempts to prepare pure serum proteins. In the search for new procedures for obtaining these substances, the author thought it worth while to try the fractionation of calf serum. From earlier investigations together with M. E. Adair and G. S. Adair (unpublished) it was known that serum from the newly born lamb and foal only contained small amounts of the ordinary globulin ($s_{20} \sim 7$). Later experiments carried out on serum from new-born calves, at different intervals after their birth, had shown that it was difficult to detect ordinary globulin on the sedimentation diagrams for serum until the calf had reached an age of one to two weeks. The sedimentation constant for the 'albumin' peak was found, however, to be exceptionally low during the first days after the birth, but gradually increased, until the normal value was reached within a few weeks.

The results obtained by Howe (1922) also demonstrated the changes taking place in the composition of calf serum with time after the birth. (At the time when the fractionation experiments, to be described below, were started, abstracts of the papers of Jameson *et al.* (1942) and San Clemente and Huddleson (1943) had not arrived at Upsala, and the note of Polson (1943) was not yet published.) All information available thus indicated differences between the compositions of the sera from the adult and the newly born animal. It was to be hoped, therefore, that some of the serum proteins might be more easily prepared from calf serum.

Experiments by the author (1944) showed that the serum from newly born calves contained only very little euglobulin (according to the definition of Howe) with $s_{20} \sim 7$. The most remarkable result was, however, that a new globulin was found with $M = 51{,}000$ and $s_{20} = 3{\cdot}3$. The name fetuin was proposed for this protein.

Fractionation Experiment No. 9. In this case the blood from several newly born calves (ages below 1 week) was pooled immediately after the slaughter and brought to the Institute, where it was treated in the usual way. As an example, the preparation will be described in somewhat greater detail.

Two litre of serum was diluted with 2 l. 0·2 M-NaCl solution which also contained sulphuric acid corresponding to 70 ml. 1 N-H_2SO_4. To this dilute serum solution, which had a *p*H of about 6, 4 M-Am_2SO_4 was added slowly through a capillary ending below the surface of the continuously stirred solution. At a concentration of '0·33 Am_2SO_4' a few inconsiderable flakes were formed in the solution and the appearance was the same even at '0·35 Am_2SO_4'. A total of 2670 ml. 4 M-Am_2SO_4 was mixed in this way with the serum. The precipitate was allowed to settle overnight at room temperature. The next morning the clear supernatant (*p*H = 5·92) was siphoned away and the rest of the mixture centrifuged strongly. The precipitate was washed twice (resuspended and centrifuged down) with 700 ml. '0·43 Am_2SO_4' and finally dissolved in 100 ml. water. The solution (volume: 242 ml.) was centrifuged clear, whereby a dark brown deposit was formed at the bottom of the centrifuge tube and a small yellow 'cream' collected at the top of the tube; both were rejected. One tenth of the clear globulin solution was dialysed against 0·2 M-NaCl until free of sulphate, giving 38 ml. of a solution with $\Delta n = 781$, viz. a total of 14·84 g. protein, subsequently called $g_1\,\varepsilon_1$.

The mother liquor from $g_1\,\varepsilon_1$ was mixed, as above, with a further 600 ml. 4 M-Am_2SO_4 resulting in a final concentration of about '0·45 Am_2SO_4'. The mixture was allowed to settle for about 3 hours and then treated in the same way as $g_1\,\varepsilon_1$. The washing was first done with 200 ml. and then with 100 ml. '0·47 Am_2SO_4'. The precipitate, $g_2\,\varepsilon_1$, was dissolved in 20 ml. (volume of solution 55 ml.) and dialysed against 0·2 M-NaCl, which resulted in 83 ml. of a solution with $\Delta n = 748$, viz. a total of 3·10 g. protein in the $g_2\,\varepsilon_1$ fraction.

By the addition of a further 730 ml. 4 M-Am_2SO_4 the salt concentration was increased to about '0·50 Am_2SO_4' and $g_3\,\varepsilon_1$ was precipitated. The procedure for $g_2\,\varepsilon_1$ was repeated, except that the wash-solution was '0·53 Am_2SO_4'. Result: $g_3\,\varepsilon_1$, 44 ml., $\Delta n = 1156, \backsim 2{\cdot}54$ g. protein.

On adding 900 ml. 4 M-Am_2SO_4 to the mother liquor no appreciable precipitate was formed. An extra 1100 ml. of ammonium sulphate solution increased the salt concentration to about '0·60 Am_2SO_4' and produced the deposit $ga\,\varepsilon_1$, which was treated in the same way as $g_2\,\varepsilon_1$ and $g_3\,\varepsilon_1$, the wash solution being '0·63 Am_2SO_4'. Result: $ga\,\varepsilon_1$, 35 ml., $\Delta n = 700, \backsim 1{\cdot}23$ g. protein.

Finally the albumin fractions were precipitated by first acidifying to *p*H 4·8, giving $a_1\,\varepsilon_1$, and then to *p*H 4·4, giving $a_2\,\varepsilon_1$ and finally solid Am_2SO_4 was added almost to saturation, giving $a_3\,\varepsilon_1$. $a_1\,\varepsilon_1$ was washed twice with '0·65 Am_2SO_4' and dissolved in 75 ml. H_2O giving 380 ml. of a grey-green solution. Some sticky, coloured deposit was discarded after centrifugation. For concentration determination, 19 ml. $a_1\,\varepsilon_1$ was dialysed against 0·2 M-NaCl. Some further coloured precipitate settled during dialysis and was thrown away after spinning the solution in a centrifuge. Result: 1/20 $a_1\,\varepsilon_1$, 26 ml., $\Delta n = 985, \backsim 1{\cdot}28$ g. protein or $a_1\,\varepsilon_1 \backsim 25{\cdot}6$ g. protein.

The protein in the wash-solution was also taken into account, the amount of substance lost in this way being determined by saturating the solutions with Am_2SO_4, dissolving the precipitated protein and dialysing the solution against 0·2 M-NaCl. The result is shown in Table 11.

According to this table, the washing had been satisfactory for the fractions $g_2\,\varepsilon_1$, $g_3\,\varepsilon_1$, and $ga\,\varepsilon_1$, whereas it had been insufficient for $g_1\,\varepsilon_1$. The great amount of protein in the washings from $a_1\,\varepsilon_1$ is probably due to the frequently observed difficulty

TABLE 11. AMOUNT OF PROTEIN IN VARIOUS FRACTIONS AND WASH-SOLUTIONS FROM CALF SERUM.

Fraction	Protein in grams	Per cent of total	Protein (in grams)	
			First wash-solution	Second wash-solution
$g_1\varepsilon_1$	14·84	25·6	0·32	0·32
$g_2\varepsilon_1$	3·10	5·4	0·14	0·02
$g_3\varepsilon_1$	2·54	4·4	0·32	0·07
$ga\,\varepsilon_1$	1·23	2·1	0·33	0·04
$a_1\varepsilon_1$	25·60	44·2	1·89	1·83
$a_2\varepsilon_1$	2·77	4·8		
$a_3\varepsilon_1$	2·54	4·4		
Wash-solutions	5·28	9·1		
Total	57.90	100·0	3·00	2·28

of tightly packing large albumin precipitates in the centrifuge tube. It may also be that the ammonium sulphate concentration was too low and part of the albumin was redissolved.

An ultracentrifugal examination of some of the wash-solutions showed that they consisted mainly of substances with s_{20} as for albumin. The wash-solutions from $g_1\varepsilon_1$ did, however, contain some fetuin, the second more than the first.

The different globulin fractions were also investigated in the ultracentrifuge and the result has been summarized in Table 12. (See also Fig. 26.)

Different attempts were made to obtain pure fetuin from $g_1\varepsilon_1$. Thus a part of the solution was dialysed against 0·02 M-NaH_2PO_4, whereupon a large amount of material

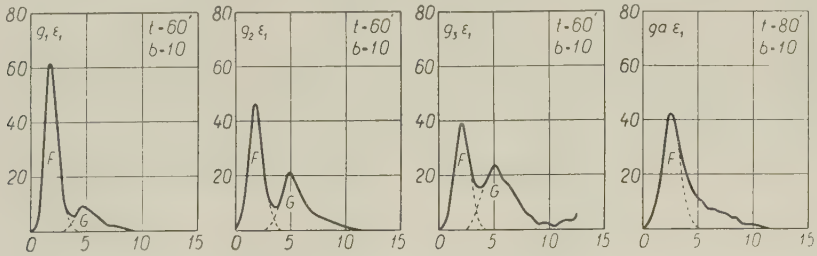

FIG. 26. Sedimentation diagrams for the globulin fractions of calf serum, showing the fetuin and the globulin peaks.

The 20-component has already sedimented to the bottom of the cell. As is evident from the diagrams, the appearance of the globulin peak indicates that much inhomogeneous material sediments together with this protein. The concentrations of the centrifuged solutions were, from left to right, 2·7, 2·6, 2·9 and 2·5 per cent. The sedimentation diagrams have all been reduced to an initial concentration of 1 per cent.

TABLE 12. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT No. 9).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$g_1\ \varepsilon_1$	14·84	547	2·87	222	40	5·94					5·89	54	10	1·48	14·6	54	10	1·48				40	5·94	0·74
$g_2\ \varepsilon_1$	3·10	524	2·92	185	35	1·10					6·15	146	28	0·86	15·9	24	5	0·15				32	0·99	0·16
$g_3\ \varepsilon_1$	2·54	578	3·11	195	34	0·85					6·23	226	39	1·00								27	0·69	0·13
$ga\ \varepsilon_1$	1·23	490					*3·50*	191	39	0·48									117	24	0·30	37	0·45	0·06
$a_1\ \varepsilon_1$	25·60																							1·28
$a_2\ \varepsilon_1$	2·77	532					3·91	210	40	1·11									166	31	0·86	29	0·80	0·14
$a_3\ \varepsilon_1$	2·54																							0·13
'Total globulin'	20·48				39	7·89							16	3·34			8	1·63				37	7·62	1·03

TABLE 13. COMPOSITION OF VARIOUS SUB-FRACTIONS FROM $g_1\varepsilon_1$.

$g_{1am}\ \varepsilon_1$	1·77	568	2·82	234	41	0·73									15·2	34	6	0·11	28	5	0·08	48	0·85	
$g_{1b}\ \varepsilon_1$	1·41	852	2·61	360	42	0·60									13·4	145	17	0·24	197	23	0·32	18	0·25	
$g_{1c}\ \varepsilon_1$	0·22	208	3·42	67	32	0·07					7·24	56	27	0·06	18·5	23	11	0·02				30	0·07	
$g_{1amA}\ \varepsilon_1$	1·02	1059	2·16	539	51	0·52									11·5	81	8	0·08	80	7	0·07	34	0·35	
do.	do.	537	2·76	222	41	0·42													150	28	0·28	31	0·32	

precipitated which, after inspection in the ultracentrifuge was found to consist of almost equal parts of fetuin and inhomogeneous material sedimenting with $s_{20} \geqq 5$. The dialysed solution was spun in the air-driven centrifuge for 4 hours at 27,000 R.P.M. A part of the upper 5/6 of the solution was used for ultracentrifugal examination after the addition of NaCl to make the solution 0·2 M. with respect to NaCl. According to the sedimentation diagrams the composition was as follows:

	Fetuin	Globulin (+ inhomogeneous material)	20-component	Material not accounted for in sedimentation diagram
Per cent	50	14	4	32

The relative concentration of the fetuin has thus increased somewhat, and that of the 20-component decreased, but the method does not seem to offer any advantages, the decrease in the heavy component being probably due to the spinning of the solution in the air-driven centrifuge.

The re-fractionation of a part of $g_1\,\varepsilon_1$ with ammonium sulphate was also attempted. The precipitates at '0·33', '0·40', and '0·50 Am_2SO_4', $g_{1a}\,\varepsilon_1$, $g_{1b}\,\varepsilon_1$ and $g_{1c}\,\varepsilon_1$ respectively, were examined in the ultracentrifuge (see Table 13). Before ultracentrifugation $g_{1a}\,\varepsilon_1$ was run 4 hours at 27,000 R.P.M. in the air-driven centrifuge, and its middle fraction $g_{1am}\,\varepsilon_1$ was used for the further experiments. The main part of this fraction was once more precipitated at '0·33 Am_2SO_4' which resulted in $g_{1amA}\,\varepsilon_1$.

In this case also the improvement in the purity of the fetuin was very small, although the appearance of the sedimentation diagrams was somewhat improved. Some runs were made on $g_{1amA}\,\varepsilon_1$ at different dilutions in order to determine the variation of s_{20} with concentration for the fetuin. The following values were obtained for fetuin.

Δn =	1059	537	265	132
s_{20} =	2·16	2·76	3·06	3·23

$$s^{\circ}_{20} = 3{\cdot}38.$$

The variation of s_{20} with the concentration is thus very pronounced.

The first fractionation experiments with calf serum showed quite a different composition of the globulin fraction as compared with that from cow serum. In addition large amounts of the new globulin fetuin were present. The attempts to purify the fetuin were not very successful, however, and other methods had to be tried.

FRACTIONATION EXPERIMENT No. 10. To 1 litre of the same serum as used in No. 9, 4 litres of the following solution was added under continuous stirring: 200 ml. 1 M-NaCl + 30 ml. 1 N-H_2SO_4 + 1620 g. Am_2SO_4 + water to give a total of 4 litres. The mixture, which thus corresponded to '0·60 Am_2SO_4', was left overnight at room temperature. The next morning the clear supernatant (*p*H 5·81) was siphoned away and the rest of the mixture centrifuged tightly down. The deposit was then suspended in 1 litre of 0·60 Am_2SO_4, stirred occasionally for some hours and finally centrifuged sharply down. The protein in the wash-solution (or extract) was precipitated with

TABLE 14. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM OBTAINED BY SUCCESSIVE EXTRACTIONS OF THE TOTAL PRECIPITATE AT '0·60 Am_2SO_4' WITH AMMONIUM SULPHATE SOLUTIONS OF DECREASING CONCENTRATION.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
e_{6m} ε_2	0·86	162	3·47	34	21	0·18					6·87	45	28	0·24		15	9	0·08	11	7	0·06	35	0·30	0·09
$e_{5a,m}$ ε_2	2·30	491	3·00	185	38	0·87						110	22	0·51	15·5	56	11	0·25				29	0·67	0·23
$e_{5a,bot}$ ε_2	0·50	507	3·16	56	11	0·06					6·49	118	23	0·11	16·5	285	56	0·28				10	0·05	0·05
e_{4b} ε_2	0·31	264	3·41	69	26	0·08					6·94	49	19	0·06					40	15	0·05	40	0·12	0·03
e_{4a} ε_2	0·57	326	3·22	83	25	0·14					6·90	63	19	0·11					48	15	0·09	41	0·23	0·06
e_{3b} ε_2	0·16	201					*3·71*	41	20	0·03	7·50	41	20	0·03					28	14	0·02	46	0·08	0·02
e_{3a} ε_2	0·50	233					*3·89*	65	28	0·14		67	29	0·14					30	13	0·07	30	0·15	0·05
e_{2b} ε_2	0·14	272					4·90	74	27	0·04									40	15	0·02	58	0·08	0·01
e_{2a} ε_2	0·75	452					4·28	247	55	0·41									152	33	0·25	12	0·09	0·08
e_1 ε_2	0·93	500					4·16	350	70	0·65									113	23	0·21	7	0·07	0·09
a_1 ε_2	2·10	484					4·32	331	68	1·43									95	20	0·42	12	0·25	0·21

solid Am_2SO_4, dissolved in water and ultimately dialysed against 0·2 M-NaCl until free of sulphate. The resulting solution was called $e_1\,\varepsilon_2$.

The precipitate that had been washed with '0·60 Am_2SO_4' was then extracted twice with 1 litre of '0·50 Am_2SO_4' in the same way as above, the process resulting in the two solutions $e_{2a}\,\varepsilon_2$ and $e_{2b}\,\varepsilon_2$.

Similar extractions were carried out with '0·45 Am_2SO_4', '0·40 Am_2SO_4', '0·35 Am_2SO_4' and finally with 0·2 M-NaCl. The corresponding solutions obtained were: $e_{3a}\,\varepsilon_2$, $e_{3b}\,\varepsilon_2$, $e_{4a}\,\varepsilon_2$, $e_{4b}\,\varepsilon_2$, $e_{5a}\,\varepsilon_2$, $e_{5b}\,\varepsilon_2$ and $e_6\,\varepsilon_2$. By an oversight $e_{5b}\,\varepsilon_2$ was discarded. The other solutions were investigated in the ultracentrifuge, but two of them were first spun in the air-driven centrifuge for four hours at 27,000 R.P.M. In both cases the top fraction was very milky and was discarded. In the one case, only the middle fraction (4/5 of the total) $e_{6,\,m}\,\varepsilon_2$, was used, the bottom fraction consisted of a massive gelatinous pellet which was very difficult to dissolve and was rejected. In the other case both the middle fraction, $e_{5a,\,m}\,\varepsilon_2$, and the bottom fraction, $e_{5a,\,bot.}\,\varepsilon_2$, were used. The result of the ultracentrifugal examination of the different solutions is summarized in Table 14. In addition the values for $a_1\,\varepsilon_2$ are given. This solution was obtained by changing the *p*H of the mother liquor from the first precipitation at '0·60 Am_2SO_4' to *p*H 5·0.

If we compare the two preparations ε_1 and ε_2, we find a higher fetuin content in the former and much more inhomogeneous material in the latter. An inspection of the sedimentation diagrams also shows nicer curves for ε_1 than for ε_2. Some further fractionations on ε_2 were tried with ammonium sulphate precipitation and with dialysis against very dilute salt solutions. Some of the attempts resulted in slightly better sedimentation diagrams, but no results were gained worthy of further mention.

Some preliminary experiments were made on the fractionation of calf serum with ethanol at low temperature according to the method of Cohn *et al.* (1940, b). The ultracentrifugal examination of the different fractions showed, however, that a large part of the sedimenting material was inhomogeneous and that this procedure for obtaining pure protein fractions was definitely inferior to the ordinary method of fractionation with salt precipitation.

During the experiments with fractionation by dialysis it was sometimes observed that the first deposit formed was less homogeneous, according to the sedimentation diagrams, than was the dialysate. In some special cases, however, for instance with certain pathological human sera, comparatively pure euglobulin fractions could be obtained. An attempt was therefore made with dialysis of the serum before the fractionation with ammonium sulphate.

TABLE 15. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT No. 11).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$eug_1\ \varepsilon_4$	0·04	124									8·3[1]	48	39	0·016					12	10	0·004	51	0·020	0·04
$eug_1'\ \varepsilon_4$	0·08	149									7·2[1]	78	52	0·042	14[1]	29	20	0·016				28	0·022	0·08
$g_1\ \varepsilon_4$	0·49	628	2·78	345	55	0·27									14·1	66	10	0·05				35	0·17	0·49
$g_2\ \varepsilon_4$	0·24	412	3·14	164	40	0·09													134	32	0·08	28	0·07	0·24
$ga_1\ \varepsilon_4$	0·14	271					4·37	149	55	0·08									74	27	0·04	18	0·02	0·14
$ga_2\ \varepsilon_4$	0·34	468					4·25	301	64	0·22									58	13	0·04	23	0·08	0·34
$a_1\ \varepsilon_4$	1·23	667					3·72	492	74	0·91												26	0·32	1·23
$a_2\ \varepsilon_4$	0·43																							0·43
'Total globulin'	0·85				42	0·36							7	0·06			8	0·07		10	0·08	33	0·28	0·85

TABLE 16. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT No. 12).

$g_1\ \varepsilon_5$	0·67	603	2·96	282	47	0·32					[1]	121	20	0·13		58	9	0·06				24	0·16	0·67
$g_2\ \varepsilon_5$	0·18	248	*3·61*	87	35	0·06					6·5[1]	104	42	0·08								23	0·04	0·18
'Total globulin'[2]	0·85				44	0·38							25	0·21			7	0·06				24	0·20	0·85
$g_1\ \varepsilon_8$	0·50	643	2·55	333	52	0·26					[1]	72	11	0·05	15·4	53	8	0·04				29	0·15	0·50
$g_2\ \varepsilon_8$	0·15	357	*3·56*	143	40	0·06					5·75 6·55	165	46	0·07								14	0·02	0·15
'Total globulin'[2]	0·65				49	0·32							19	0·12			6	0·04				26	0·17	0·65

[1] Extremely inhomogeneous.

[2] Except the euglobulins.

Fractionation Experiment No. 11. 100 ml. of calf serum (1 week old) was diluted with a mixture of 4·2 ml. 1 N-H_2SO_4 and 96 ml. H_2O. (Final *p*H about 5·0). The solution was then dialysed against 0·01 M-NaH_2PO_4 for about 20 hours, when a large yellow precipitate formed. This was first extracted with 6 ml. of 0·2 M-NaCl and then with 0·2 M-Na_2HPO_4, when most of the remaining precipitate went into solution. The two solutions were $eug_1\,\varepsilon_4$ and $eug'_1\,\varepsilon_4$ respectively.

The *p*H of the dialysed solution of serum was changed from 5·2 to about 6·4 and ammonium sulphate was added to '0·40 Am_2SO_4', resulting in $g_1\,\varepsilon_4$. No precipitation was observed at '0·33 Am_2SO_4'. The other precipitations were made at '0·50 Am_2SO_4' ($g_2\,\varepsilon_4$), '0·60 Am_2SO_4' ($ga_1\,\varepsilon_4$), '0·60 Am_2SO_4, *p*H 5·0' ($ga_2\,\varepsilon_4$), *p*H 4·7 ($a_1\,\varepsilon_4$) and finally by adding solid Am_2SO_4 ($a_2\,\varepsilon_4$).

Ultracentrifugal examination of the different fractions showed $eug_1\,\varepsilon_4$ and $eug'_1\,\varepsilon_4$ to be extremely inhomogeneous, whereas $g_1\,\varepsilon_4$ was so far the purest fetuin solution obtained. The next fraction, $g_2\,\varepsilon_4$, as regards purity, very much resembled the best of the foregoing fetuin fractions. The result is summarized in Table 15.

By suitable dialysis it thus seems possible to remove a part of the very disturbing inhomogeneous material simultaneously as most of the euglobulin (Howe) is withdrawn from the serum. A similar dialysis of the serum before the precipitation with ammonium sulphate was then considered so advantageous that it was used in some of the following fractionation experiments.

Fractionation Experiment No. 12. A part of the same serum as in No. 11 was dialysed in a similar way as above. Four aliquots of 100 ml. of the dialysed solution were adjusted to *p*H 5, 6, 7 and 8 and precipitated at '0·40 Am_2SO_4' (preparations named ε_5, ε_6, ε_7 and ε_8, respectively) the amount of protein in each fraction from ε_5 to ε_8 being 0·67, 0·47, 0·49 and 0·50. By increasing the salt concentration to '0·50 Am_2SO_4' the corresponding protein values were 0·18, 0·25, 0·22 and 0·15. A determination of the supernatants from the first precipitations showed that the addition of the ammonium sulphate had changed the *p*H of the solutions considerably, and most in the later ones. For ε_5 it had changed only from 5·0 to 5·11, but for ε_8 from 8·0 to 5·85. The results of the ultracentrifugal investigation of the two fractions from ε_5 and ε_8 are summarized in Table 16.

The results show that the first fraction is much purer with respect to fetuin than the corresponding fractions in ε_1 and ε_2 but less pure than $g_1\,\varepsilon_4$. Of the two fractions, $g_1\,\varepsilon_8$ contains a smaller amount of fetuin, but it seems to be of a slightly purer quality than $g_1\,\varepsilon_5$.

During this experiment it was noticed that the fractions g_1 and g_2 had a definite yellow colour in the preparations ε_5 and ε_6, but were almost colourless in ε_7 and ε_8. This is undoubtedly connected with the serum lipids and the manner in which they are associated with the

respective proteins, or with different powers of the lipids, at different *p*H values, to dissolve the pigments of serum (in the present case mainly carotenes?). From more recent experiments, however, it seems that the substances which colour the globulin fractions precipitated around *p*H 5 are connected with some coarse disperse material that may comparatively easily be removed from the protein solution by spinning at high speed in the air-driven centrifuge.

Fractionation Experiment No. 13. For this experiment serum from a calf, about six months old, was used. One litre of serum was acidified to *p*H 5 and dialysed against 0·01 M-NaH_2PO_4 for two days in the cold room. After the deposit had been centrifuged down the supernatant was adjusted to *p*H 5·0 and diluted to 2 litres, whereby a new deposit formed and was also centrifuged down. Both deposits were discarded, as a test dialysis on a small amount of serum, carried out in the same manner as before had shown that the precipitated protein appeared very inhomogeneous in the ultracentrifuge.

The dialysed serum was then divided into two aliquots of 1 litre; and the *p*H of one, ε_9, was kept at 5·0 and that of the other, ε_{10}, was changed to 8·0. No precipitate was produced in any of the solutions by adding ammonium sulphate to '0·33 Am_2SO_4', but at '0·40 Am_2SO_4' heavy deposits were obtained. Each precipitate was washed with 100 ml. of a wash-solution of the concentration given in Table 17. The amount of protein lost in some of these solutions are also given.

Table 17.

Fraction	Precipitated at Am_2SO_4	*p*H of supernatant	Protein in grams	Washed with Am_2SO_4	Grams of protein in wash-solution
$g_1\,\varepsilon_9$	'0·40'	5·78	0·94	'0·43'	
$g_2\,\varepsilon_9$	'0·45'	5·90	1·34	'0·48'	0·07
$g_3\,\varepsilon_9$	'0·50'	6·03	0·45	'0·53'	0·07
$g_1\,\varepsilon_{10}$	'0·40'	5·78?	0·89	'0·43'	0·11
$g_2\,\varepsilon_{10}$	'0·45'	6·84	0·77	'0·48'	0·14
$g_3\,\varepsilon_{10}$	'0·50'	6·86	0·34	'0·53'	0·04

Ultracentrifugation of the different fractions showed much more uniform globulin peaks than in ε_5 and ε_8. This is undoubtedly due to the fact that the serum was taken this time from a calf six months old. Fig. 27 illustrates the difference between $g_2\,\varepsilon_5$, $g_2\,\varepsilon_8$ and the corresponding two fractions in ε_{10}.

The ultracentrifugal results from ε_9 and ε_{10} have been summarized in Table 18.

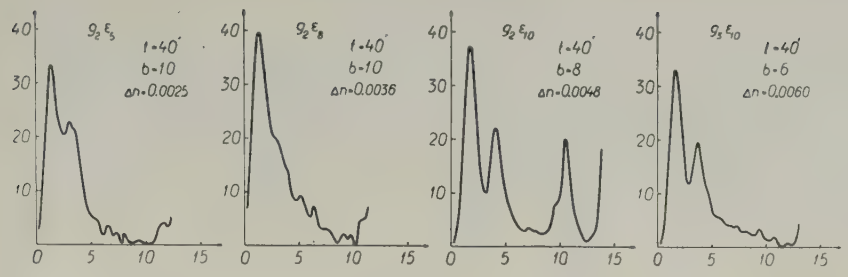

FIG. 27. Sedimentation diagrams for the serum globulin fractions precipitated between '0·40' and '0·50 Am_2SO_4' from a new-born calf (left) and from a six months old calf (right).

All the diagrams have been reduced to the same protein concentration, $\Delta n = 200$.

FRACTIONATION EXPERIMENT No. 14. The serum was taken this time from a calf about two weeks old. Two different methods of preparation were tried. In the first, ε_{12}, the precipitation was alternately carried out at *p*H 5 and *p*H 7, whereas in the second, ε_{13}, no adjustment of the hydrogen ion concentration was made. In the first preparation the ammonium sulphate solutions were adjusted to *p*H 5 and *p*H 7, respectively, before use (denoted by 'Am_2SO_4, *p*H 5' and 'Am_2SO_4, *p*H 7', respectively).

500 ml. of serum was diluted with an equal volume of 0·2 M-NaCl and the mixture adjusted to *p*H 5·0. Addition of ammonium sulphate in the usual way to '0·33 Am_2SO_4, *p*H 5' produced the precipitate $g_1^5\,\varepsilon_{12}$. This was washed with 100 ml. '0·37 Am_2SO_4, *p*H 5'. The *p*H of the mother liquor was changed to 7, but, since no deposit was formed, it was changed back to *p*H 5 again and 90 ml. 'Am_2SO_4, *p*H 5' was added. The precipitate $g_2^5\,\varepsilon_{12}$ was washed twice with 100 ml. '0·43 Am_2SO_4, *p*H 5'. When the *p*H of the mother liquor was altered to 7 a small deposit, $g_2^7\,\varepsilon_{12}$, formed which was washed with '0·43 Am_2SO_4, *p*H 7'.

Addition of 80 ml. Am_2SO_4, *p*H 7 to the mother liquor from $g_2^7\,\varepsilon_{12}$ produced the precipitate $g_3^7\,\varepsilon_{12}$. On centrifuging this down, one of the centrifuge vessels cracked and approximately one fourth of the total deposit was spoiled and about one third of the mother liquor was lost. In Table 19 the values for $g_3^7\,\varepsilon_{12}$ and the following fractions have been corrected for this loss. This means, however, that they can only be considered as approximate. By changing the *p*H of the mother liquor to 5·0 the precipitate $g_3^5\,\varepsilon_{12}$ was formed. It was washed with 100 ml. 0·45 Am_2SO_4, *p*H 5.

Adjustment of the *p*H of the mother liquor to 7 and addition of 231 ml. 'Am_2SO_4, *p*H 7' produced $g_4^7\,\varepsilon_{12}$, and this was washed with 100 ml. '0·55 Am_2SO_4, *p*H 7'. When the *p*H was changed back to 5, a heavy precipitate, $g_4^5\,\varepsilon_{12}$, was formed. The washing was carried out with 200 ml. '0·55 Am_2SO_4, *p*H 5', and during this procedure one of the centrifuge tubes again cracked and about half of the precipitate was lost.

Neutralizing the mother liquor and adding 154 ml. 'Am_2SO_4, *p*H 7' caused no precipitation. When the *p*H was brought back to 5, $ga_1^5\,\varepsilon_{12}$ was obtained. It was washed with 100 ml. '0·60 Am_2SO_4, *p*H 5'.

TABLE 18. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT NO. 13).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
g_1 ε_9	0·94																							
g_2 ε_9	1·34	716	2·66	190	26	0·35					5·79	263	37	0·50	13·7	154	22	0·29				15	0·20	
g_3 ε_9	0·45	523	3·22	145	28	0·13					6·35	234	45	0·20		9	2	0·01	61	11	0·05	14	0·06	
g_1 ε_{10}	0·89	615	3·01	46	8	0·07					6·00	223	36	0·32	16·8	38	6	0·05				50	0·45	
g_2 ε_{10}	0·77	484	2·85	156	32	0·25					6·22	127	26	0·20	15·2	124	26	0·20				16	0·12	
g_3 ε_{10}	0·34	595	2·99	215	36	0·12					6·04	156	26	0·09					158	27	0·09	11	0·04	
'Total globulin'[1]	2·00				22	0·44							31	0·61			13	0·25		4	0·09	30	0·61	

TABLE 19. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT NO. 14).

g_1^5 ε_{12}	3·45	406	3·22	109	27	0·93						43	11	0·38	16·3	37	9	0·31				53	1·83	0·69
g_2^5 ε_{12}	0·84	557	3·15	161	29	0·24					6·76	157	28	0·24	18·0	24	4	0·03				39	0·33	0·17
g_2^7 ε_{12}	0·67	640	2·83	83	13	0·09	*5·02*	73	11	0·07	6·59	306	48	0·32					65	10	0·07	18	0·12	0·13
g_3^7 ε_{12}	0·41	481	3·11	75	15	0·06	4·24	28	6	0·02	6·71	172	36	0·15					52	11	0·05	32	0·13	0·08
g_3^5 ε_{12}	1·35	625	3·00	137	22	0·30	4·3	101	16	0·22	*8·9*	26	4	0·05								58	0·78	0·27
g_4^7 ε_{12}	0·90	633	*3·28*	180	28	0·25	4·6	92	15	0·14	7·11	108	17	0·15								40	0·36	0·18
g_4^5 ε_{12}	6·68	445	2·95	9	2	0·13	4·34	315	71	4·74												27	1·81	1·34
ga_1^5 ε_{12}	3·07																							0·61
a_1 ε_{12}	1·50																							0·30
a_3 ε_{12}	1·06																							0·21
'Total globulin'[2]	7·62				24	1·87			6	0·45			17	1·29			5	0·34		1	0·12	47	3·55	1·52

$(g_1^7\ \varepsilon_{12})_{ac,c}$	0·043	426	3·23	107	25	0·011								41	9	0·004				37	9	0·004	57	0·024	[3]
do.	0·029	285	3·30	90	32	0·009					7·47	15	5	0·002						89	31	0·009	32	0·009	[4]

TABLE 20. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT NO. 14).

$g_1\ \varepsilon_{13}$	0·75	487	2·75	38	8	0·06					6·09	278	57	0·43									35	0·26	0·19
$g_2\ \varepsilon_{13}$	1·09	869	2·46	358	41	0·45					5·25	130	15	0·16	11·0	64	7	0·08					37	0·40	0·27
$g_3\ \varepsilon_{13}$	1·25	584	2·70	325	55	0·69					5·81	56	10	0·13	14·3	74	13	0·16	6	1	0·01		21	0·26	0·31
$g_4\ \varepsilon_{13}$	0·71	530	*3·14*	268	50	0·35					7·66	95	18	0·13									32	0·23	0·18
$ga_1\ \varepsilon_{13}$	0·56	531					4·28	255	48	0·27													52	0·29	0·14
$a_1\ \varepsilon_{13}$	6·72	521					4·23	349	67	4·50													33	2·22	1·68
$a_2\ \varepsilon_{13}$	1·04																								0·26
$a_3\ \varepsilon_{13}$	0·40																								0·10
$a_4\ \varepsilon_{13}$	0·16	155					*3·41*	94	61	0·10									61	39	0·06				0·04
'Total globulin'	3·80				41	1·55							22	0·85			7	0·24			0·01		30	1·15	0·95
$(g_3\ \varepsilon_{13})_1^5$	0·48	484	2·64	282	58	0·278									15·4	40	8	0·039	38	8	0·038		26	0·125	
$(g_3\ \varepsilon_{13})_2^5$	0·17	611	2·98	228	37	0·063					6·06	71	12	0·020	14·5	124	20	0·034					31	0·053	
$(g_3\ \varepsilon_{13})_3^7$	0·14	468	3·19	135	29	0·040					6·59	102	22	0·031	16·3	67	14	0·020					35	0·049	
$(g_3\ \varepsilon_{13})_4^7$	0·08	333	*3·35*	102	31	0·024					*6·01*	25	7	0·006	16·7	30	9	0·007	21	6	0·005		47	0·038	
$(g_3\ \varepsilon_{13})_5^7$	0·04	217	*3·45*	70	32	0·013					*5·23*	30	14	0·006									54	0·021	
'Total globulin'	0·91				46	0·418							7	0·063			11	0·100		5	0·043		31	0·286	

[1] Except the euglobulins. [2] Except $g_4^5\ \varepsilon_{12}$. [3] Dissolved in 0·2 M-NaCl. [4] Dissolved in 0·2 M-Na_2HPO_4.

The addition of 196 ml. 'Am_2SO_4, *p*H 7' produced no precipitate either at *p*H 7 or at *p*H 5·1. When the solution was further acidified to *p*H 4·7, a new precipitate $a_1\varepsilon_{12}$ was produced. A change of the *p*H to 4·1 did not give any deposit. By saturating the remaining mother liquor with ammonium sulphate, $a_2\varepsilon_{12}$ was finally obtained.

The large fractions g_4^5 and ga_1^5 seem to indicate either that the ammonium sulphate concentration, for some unknown reason, has been larger than expected, or that the *p*H has been lower than 5·0. It must be emphasized once more, however, that because of the cracking of the centrifuge tubes the values for the different fractions here should be considered as mere estimates.

The results of the ultracentrifugal investigation of the different fractions are summarized in Table 19. At the end of the table, the results are given for two runs on a globulin fraction which had been extracted with acetone and ether according to the method of Blix (1941). In both cases 60 mg. of the dried product was mixed with 2 ml. of solution. In the sodium chloride solution more was dissolved ($\mathcal{A}n = 426$) than in the disodium phosphate solution ($\mathcal{A}n = 285$). The excess must, after all, have been due to coarse disperse material, since the two runs gave the same amount of substance as that recorded in the sedimentation diagram. For further details about the extraction *etc.*, *cf.* p. 128.

For comparison 400 ml. of the same serum was fractionated in the usual manner. Precipitates were successively taken at the following concentrations of saturated ammonium sulphate: 0·33, 0·39, 0·43, 0·48, and 0·57 giving the corresponding fractions $g_1\varepsilon_{13}$ to $g_4\varepsilon_{13}$ and $ga\,\varepsilon_{13}$. The albumins were then precipitated at *p*H 5·0, 4·7 and 4·1, and finally solid ammonium sulphate was added to the mother liquor to precipitate the last albumin fraction $a_4\varepsilon_{13}$. The precipitates were washed with 100 ml. Am_2SO_4 of the following concentrations: '0·37', '0·43', '0·47', '0·53' and '0·65', respectively. In this case also the values for the amount of protein for the fractions from $ga\,\varepsilon_{13}$ onwards had to be corrected for the loss due to a cracked centrifuge tube. The results from the ultracentrifugal investigation of the different fractions have been summarized in Table 20. The same table also contains the result from a further fractionation of $g_3\varepsilon_{13}$ with ammonium sulphate.

If we compare the Tables 19 and 20, the most striking result is that in ε_{12} the fetuin is mainly precipitated at *p*H 5 and the globulin at *p*H 7. In ε_{12} the maximum precipitation of fetuin takes place at '0·33 Am_2SO_4', but in ε_{13} this occurs at '0·43 Am_2SO_4'. It is thus possible, by changing the *p*H to 5, to precipitate the fetuin at a lower ammonium sulphate concentration than usual. By precipitating alternately at *p*H 5 and 7 the relative amount of fetuin is higher in the former case and lower in the latter; in the case of globulin the op-

posite holds good. There is one drawback, however, for the amount of inhomogeneous material which cannot be accounted for in the sedimentation diagram amounts in ε_{12} to about twice as much as in ε_{13}. This is undoubtedly due to some change in the protein molecules in the more acid solution. From the Tables 19 and 20 it is also evident that the relative amounts of material which have undergone this degradation are considerably higher in the fractions at *p*H 5 than in those at *p*H 7. In the re-fractionated $g_3\,\varepsilon_{13}$ there is a distinct increase in the amount of this material with increase in ammonium sulphate concentration. From the re-fractionation experiment it is also seen that, when pure fetuin is wanted, the first precipitate in the new fractionation gives the best result.

Fractionation Experiment No. 15. For this experiment 500 ml. serum from a calf, two to three days old, was used. The fractionation was made in a manner similar to that with ε_{12}. The fractions were taken at '0·33', '0·37', '0·40', '0·45', '0·50', and '0·55 Am_2SO_4': No precipitation occurred at '0·60 Am_2SO_4' until the *p*H was changed to 4·7. At *p*H 4·1 $a_2\,\varepsilon_{14}$, and at full saturation $a_3\,\varepsilon_{14}$ were obtained. Attempts were made to get precipitates at '0·45 Am_2SO_4', but with no success. The washings were made with 100 ml. of solutions having concentrations higher by '0·05 Am_2SO_4' than the corresponding precipitates. Three of the fractions were re-fractionated with ammonium sulphate. Some of the fractions and sub-fractions from ε_{14} were examined in the ultracentrifuge and the results have been summarized in Tables 21—22.

The trend in the figures very much resembles that in Table 19, the main difference being that the fetuin is mainly precipitated at '0·37' and '0·40 Am_2SO_4' in ε_{14} but at '0·33 Am_2SO_4' in ε_{12}. The very distinct difference between the precipitates at *p*H 5 and 7 is demonstrated in Fig. 28 with the corresponding sedimentation diagrams for four successive fractions from ε_{14}.

The result of the re-fractionation is by no means so encouraging as might have been expected from the first fractionation illustrated in Fig. 28. The best result is obtained with $(g_2^7\,\varepsilon_{14})_1^7$, but $g_2^7\,\varepsilon_{14}$ was already comparatively pure globulin as seen from Fig. 28. In the case of fetuin the purification is less good. For $g_2^5\,\varepsilon_{14}$ the best result is obtained with $(g_2^5\,\varepsilon_{14})_2^5$ if it is taken into account that the 20-component is comparatively easy to remove by high-speed centrifugation or by dialysis. On the other hand, the amount of fetuin in $(g_2^5\,\varepsilon_{14})_2^5$ is only a small fraction of that originally present in $g_2^5\,\varepsilon_{14}$. For $g_3^5\,\varepsilon_{14}$ the conditions are slightly better since the normal globulin is absent from $(g_3^5\,\varepsilon_{14})_3^5$.

TABLE 21. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM (FRACTIONATION EXPERIMENT No. 15).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram				Inhomogen. material not in sedimentation diagram		Grams of protein per 100 ml. of serum
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.		%	g.	
$g_1^5\ \varepsilon_{14}$	0·46	507	2·99	103	20	0·09					5·97	118	23	0·10	16·0	32	6	0·03	127	25	0·12		26	0·12	0·09
$g_2^5\ \varepsilon_{11}$	1·61	537	3·01	170	32	0·51					6·49	64	12	0·19	15·8	70	13	0·21	49	9	0·15		34	0·55	0·32
$g_2^7\ \varepsilon_{14}$	0·69	617	3·52	27	4	0·03					6·26	340	55	0·38									41	0·28	0·14
$g_3^5\ \varepsilon_{14}$	1·60	596	2·96	214	36	0·58					6·13	40	7	0·11	17·0	46	8	0·13	93	15	0·24		34	0·54	0·32
$g_5^7\ \varepsilon_{14}$	0·70	689	2·90	318	46	0·32					5·77	194	28	0·20									26	0·18	0·14
$g\alpha_1^5\ \varepsilon_{14}$	0·44																								0·09
$a_1\ \varepsilon_{14}$	9·14																								1·85
$a_2\ \varepsilon_{14}$	1·62																								0·32
$a_3\ \varepsilon_{14}$	2·03																								0·41
'Total globulin'	5·06				30	1·53							20	0·98			7	0·37		10	0·51		33	1·67	1·01

TABLE 22. COMPOSITION OF DIFFERENT SUB-FRACTIONS FROM CALF SERUM.

$(g_2^5\ \varepsilon_{14})_1^5$	0·76	578	*3·38*	140	24	0·182					7·32	49	9	0·069	16·1	72	12	0·091					55	0·418	
$(g_2^5\ \varepsilon_{14})_1^7$	0·07																								
$(g_2^5\ \varepsilon_{14})_2^5$	0·16	468	2·80	164	35	0·056					6·65	33	7	0·011	17·0	105	22	0·035					36	0·058	
$(g_2^5\ \varepsilon_{11})_3^7$	0·21	515	2·67	229	45	0·095					6·08	68	13	0·027	15·1	86	17	0·036	17	3	0·006		22	0·046	
$(g_2^5\ \varepsilon_{14})_4^7$	0·07																								
$(g_2^7\ \varepsilon_{11})_1^5$	0·14	643	3·28	12	2	0·003					6·59	272	42	0·059	15·5	25	4	0·005					52	0·073	
$(g_2^7\ \varepsilon_{14})_1^7$	0·14	204					4·05	4	2	0·003	6·98	176	86	0·120									12	0·017	
$(g_2^7\ \varepsilon_{14})_2^5$	0·09																								

$(g_3^5\ \varepsilon_{14})_1^5$	0·41	436	3·24	73	17	0·070									19·1	45	10	0·041	84	19	0·078	54	0·221	
$(g_3^5\ \varepsilon_{14})_2^7$	0·05																							
$(g_3^5\ \varepsilon_{14})_3^7$	0·19	483	2·40	253	52	0·099					5·3	55	11	0·021	15·4	51	11	0·021				26	0·049	
$(g_3^5\ \varepsilon_{14})_3^5$	0·12	515	2·64	255	50	0·060									15·5	47	9	0·011				41	0·049	
$(g_3^5\ \varepsilon_{14})_4^7$	0·05																							
$(g_3^5\ \varepsilon_{14})_4^5$	0·06																							

TABLE 23. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM OBTAINED AT LOW TEMPERATURE.

$g_1^5\ \varepsilon_{16}$	0·08	201	*3·92*	12	6	0·005					7·02	80	40	0·032	18·3	9	4	0·003				50	0·040	0·04
$g_1^7\ \varepsilon_{16}$	0·21	556	*3·56*	37	7	0·015					6·59	320	57	0·120					29	5	0·010	31	0·065	0·10
$g_2^5\ \varepsilon_{16}$	0·15	422	3·18	44	10	0·015					6·69	161	38	0·057	15·7	26	6	0·009	57	14	0·021	32	0·048	0·08
$g_2^7\ \varepsilon_{16}$	0·11	326	3·58	20	6	0·007					6·76	209	64	0·070	13·8	13	4	0·004				26	0·029	0·05
$g_3^7\ \varepsilon_{16}$	0·15	416	3·47	53	13	0·020					6·98	201	48	0·072	22·4	45	11	0·016	29	7	0·010	21	0·032	0·08
$g_3^5\ \varepsilon_{16}$	0·60	627	2·88	227	36	0·216					6·05	93	15	0·090	14·5	89	14	0·084				35	0·210	0·30
$g_4^7\ \varepsilon_{16}$	0·23	468	2·75	167	36	0·083					6·18	88	19	0·044	15·8	56	12	0·027	44	9	0·021	24	0·055	0·12
$g_4^5\ \varepsilon_{16}$	0·27	357	2·70	185	52	0·140	*3·80*	40	11	0·030					17·9	11	3	0·008				34	0·092	0·13
$g_5^5\ \varepsilon_{16}$	0·11	381	2·90	200	53	0·058					5·91	68	18	0·020	17·5	5	1	0·001				28	0·031	0·05
$ga_1^7\ \varepsilon_{16}$	0·12	287	3·24	92	32	0·038					6·23	24	8	0·010	18·8	11	4	0·005	23	8	0·010	48	0·057	0·06
$ga_1^5\ \varepsilon_{16}$	0·29	396	2·90	28	7	0·020	3·99	189	48	0·139									21	5	0·015	40	0·116	0·14
$ga_2\ \varepsilon_{16}$	0·40	381		23	6	0·024	4·07	245	64	0·256												30	0·120	0·20
$a_1\ \varepsilon_{16}$	2·98	595					3·44	426	72	2·146												28	0·834	1·49
$a_2\ \varepsilon_{16}$	0·78	381					*3·22*	198	52	0·406									45	12	0·093	36	0·281	0·39
$a_3\ \varepsilon_{16}$	0·29	365					*3·63*	127	35	0·102					16·7	19	5	0·014	26	7	0·020	53	0·154	0·14
'Total globulin'	1·91				29	0·559			2	0·030			26	0·505			8	0·152		3	0·062	32	0·602	0·95
'Total protein'	6·77				9	0·641			45	3·079			8	0·515			3	0·171		3	0·200	32	2·164	3·37

TABLE 24. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$g_1^5\ \varepsilon_{17}$	0·22	310	*3·65*	64	20	0·044					*8·13*	99	32	0·070	18·1	24	8	0·018				40	0·088	0·11
$g_1^7\ \varepsilon_{17}$	0·31	507	2·85	35	7	0·022					6·27	323	64	0·198	13·8	7	1	0·003				28	0·087	0·15
$g_2^5\ \varepsilon_{17}$	0·96	532	2·88	223	42	0·403						76	14	0·135	15·8	63	12	0·115				32	0·307	0·48
$g_3^7\ \varepsilon_{17}$	0·20	460	3·02	63	14	0·028					6·32	241	52	0·104	20·3	21	5	0·010				29	0·058	0·10
$g_4^7\ \varepsilon_{17}$	0·23	310	2·96	141	46	0·106					6·76	39	13	0·030	18·0	20	6	0·014	16	5	0·011	30	0·069	0·12
$g_1^5\ \varepsilon_{17}$	0·37	436	2·77	147	34	0·126	4·47	135	31	0·115	6·47	55	13	0·048		62	14	0·052				8	0·029	0·18
$ga_1^7\ \varepsilon_{17}$	0·12																							0·06
$ga_1^5\ \varepsilon_{17}$	0·15																							0·08
$ga_2^5\ \varepsilon_{17}$	0·22																							0·11
$a_1\ \varepsilon_{17}$	3·26																							1·63
$a_2\ \varepsilon_{17}$	0·54																							0·27
$a_3\ \varepsilon_{17}$	0·18																							0·09
'Total globulin'	2·29				32	0·729			5	0·115			26	0·585			9	0·212			0·011	28	0·638	1·14

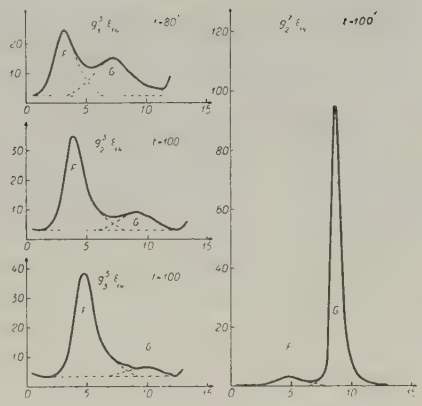

FIG. 28. Sedimentation diagrams for the globulin fractions from calf serum precipitated between '0·33' and '0·40 Am_2SO_4'.

All diagrams have been reduced to the same protein concentration ($\Delta n = 200$).

The main part of the fetuin is precipitated at *p*H 5 (left-hand diagrams) while almost pure globulin is precipitated at *p*H 7 (right-hand diagram).

From the re-fractionation experiments it seems likely that very little can be gained by a mere repetition of the procedure used in the first fractionation. It is to be expected that an improved separation may be attained by using the scheme $g_1^7 \rightarrow g_1^5 \rightarrow g_2^7 \rightarrow g_2^5 \rightarrow g_3^7 \rightarrow g_3^5$, *etc.*, especially if the precipitations at *p*H 7 are carried out in the cold room ($\sim + 5°$ C), while those at *p*H 5 are made at room temperature ($\sim + 18°$ C). By this variation in temperature the precipitation of the fetuin should be further favoured at *p*H 5, whereas it should be suppressed at *p*H 7, since it has been found that the fetuin is more soluble in the cold (see pp. 104 and 109). The author hopes to be able to illustrate this in a future paper.

The serum used for ε_{14} was subjected to two further fractionations, ε_{16} and ε_{17}. The first, ε_{16}, was carried out in the cold room ($+2°$ to $+4°$ C) in order to demonstrate whether some advantage might be gained by working at lower temperatures. The second was made as a mere repetition of ε_{14} in order to give an idea of the reproducibility of the fractionation method. The amounts of protein, per 100 ml. of serum, in the individual fractions are given in Table 25.

It appears from the table that in the preparation carried out at the lower temperature it is necessary to use somewhat higher ammonium sulphate concentration for precipitating the different globulin fractions. At about '0·45 to 0·50 Am_2SO_4' all three preparations have yielded nearly the same total amount of protein.

Comparison of the fractions ε_{14} and ε_{17} shows that the agreement between the individual fractions in the two preparations is very poor, although the general trend in the values for the total protein is more

TABLE 25. AMOUNTS OF PROTEIN, PER 100 ML. OF SERUM, IN THE VARIOUS FRACTIONS FROM CALF SERUM.

Two of the fractionations, ε_{14} and ε_{17}, were carried out at room temperature, while the third, ε_{16}, was made at low temperature.

Preparation	Individual fraction			Total amount of precipitated protein		
	ε_{14}	ε_{16}	ε_{17}	ε_{14}	ε_{16}	ε_{17}
g_1^5	0·09	0·04	0·11	0·09	0·04	0·11
g_1^7	0	0·10	0·15	0·09	0·14	0·26
g_2^5	0·32	0·08	0·48	0·41	0·22	0·74
g_2^7	0·14	0·05	0	0·55	0·27	0·74
g_3^7	0	0·08	0·10	0·55	0·35	0·84
g_3^5	0·32	0·30	0	0·87	0·65	0·84
g_4^7	0	0·12	0·12	0·87	0·77	0·96
g_4^5	0	0·13	0·18	0·87	0·90	1·14
g_5^7	0·14	0	0	1·01	0·90	1·14
g_5^5	0	0·05	0	1·01	0·95	1·14
ga_1^7	0	0·06	0·06	1·01	1·01	1·20
ga_1^5	0·09	0·14	0·08	1·10	1·15	1·28
ga_2^7	0	0	0·11	1·10	1·15	1·39
ga_2^5	0	0·20	0	1·10	1·35	1·39
a_1	1·85	1·49	1·63	2·95	2·84	3·02
a_2	0·32	0·39	0·27	3·27	3·23	3·29
a_3	0·41	0·14	0·09	3·68	3·37	3·38

nearly the same. This variation in the figures for the individual fractions is probably due to small differences in temperature, *p*H and salt concentration, or in the time allowed for the different precipitates to develop.

In the preparations at low temperature, it was not found necessary to add the ammonium sulphate slowly through a capillary. The two solutions could be mixed at once, and they would remain clear for a short period. On standing, the precipitate would gradually develop. Another difference was observed between ε_{14} and ε_{17} on the one hand and ε_{16} on the other. All the first fractions of the former were strongly coloured, especially those at *p*H 5, whereas the corresponding fractions from ε_{16} were very slightly coloured or almost colourless.

The different fractions from ε_{16} and ε_{17} were also examined in the ultracentrifuge. The results from these experiments have been summarized in Tables 23 and 24.

A comparison of Tables 21, 23 and 24 reveals that the lower amounts of globulin in the first fractions from ε_{16} must be due to a higher solubility of the fetuin at the lower temperature. It is quite clear that the relative amounts of fetuin in all the first fractions from ε_{16} are much lower than in the corresponding fractions from ε_{14} and ε_{17}, whereas the relative amounts of globulin are very much the same in all three cases.

Fractionation Experiment No. 16. It was thought worth while to attempt a repetition of Fractionation Experiment No. 10 with some modifications. One set of fractionations, ε_{19}, was carried out in the cold room ($+2°$ to $+4°$ C), the other, ε_{18}, at room temperature ($\sim 20°$ C). Some of the extractions were made at *p*H 7, ε_{18A} and ε_{19A}, the others at *p*H 5, ε_{18B} and ε_{19B}.

In each preparation 400 ml. of serum from a calf, two to three days old was diluted with 400 ml. 0·2 M-NaCl. To this mixture, 1200 ml. 4 M-Am_2SO_4 was added very slowly through a capillary under constant stirring which was continued overnight. On the following morning the deposits, at the two temperatures were centrifuged tightly down, each resuspended in 400 ml. '0·60 Am_2SO_4', left with stirring for some time, and finally centrifuged tightly down. This procedure was repeated twice. By addition of solid ammonium sulphate, the protein in these wash-solutions was precipitated, centrifuged down, dissolved in water and finally dialysed against 0·2 M-NaCl*.[1] The three solutions were pooled before the precipitation, and the dialysed solutions were called $e_{1abc}\,\varepsilon_{18}$ and $e_{1abc}\,\varepsilon_{19}$ respectively. Each of the two precipitates was then divided into two aliquots, one being extracted at *p*H 7, the other at *p*H 5. The extractions were carried out as described above with ammonium sulphate solutions having the proper *p*H and temperature. 200 ml. of solution was used each time and the procedure was repeated until solid ammonium sulphate added to the last extract did only produce a faint precipitate. In the Tables 26 to 29, the number of extractions is indicated by the number of letters after the number of the extract. Thus $e_{4abcd}\,\varepsilon_{18A}$ consists of the pooled solutions from four extractions with '0·40 Am_2SO_4, *p*H 7' at room temperature. Extractions were carried out with the solutions '0·50', '0·45', '0·40', '0·37', '0·33 Am_2SO_4' and finally 0·2 M-NaCl, the resulting protein solutions being e_2 to e_7.

[1] By 0·2 M-NaCl* is meant, in the following account, a solution which, besides being 0·2 molar with respect to NaCl, also contains 0·01 M-$KBrO_3$ and 0·02 M-NaF. The two salts have been added in an attempt to reduce enzymic activity in these protein solutions. According to investigations by Jörgensen (1935) bromates exert a strong inhibitory action on proteolytic enzymes, whereas fluorides act as inhibitors for many lipases.

TABLES 26—30. COMPOSITION OF DIFFERENT FRACTIONS FROM CALF SERUM OBTAINED BY SUCCESSIVE EXTRACTIONS OF THE TOTAL PRECIPITATE AT '0·60 Am_2SO_4' WITH AMMONIUM SULPHATE SOLUTIONS OF DECREASING CONCENTRATION.

TABLE 26. Extractions Performed at *p*H 7 and Room Temperature (~ 20° C).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
e_{7a} ε_{18A}	0·16	162	2·55	11	7	0·011					7·59	53	33	0·053					12	7	0·011	53	0·085	0·04
e_{6ab} ε_{18A}	0·53	548	3·44	76	14	0·074					6·13	188	34	0·180	16·8	22	4	0·021				48	0·255	0·13
e_{5abc} ε_{18A}	0·71	318	2·98	107	34	0·241					6·79	88	28	0·199	18·1	43	13	0·092				25	0·178	0·18
e_{4abcd} ε_{18A}	1·14	318	3·05	159	50	0·570					6·89	101	32	0·365	17·4	32	10	0·114				8	0·091	0·28
e_{3ab} ε_{18A}	0·24	287	*3·49*	110	38	0·091					6·93	71	25	0·060								37	0·089	0·06
e_{2ab} ε_{18A}	0·27	420					*3·81*	176	42	0·113									49	12	0·032	46	0·125	0·07
e_{1abc} ε_{18A}	0·35	658					4·03	383	58	0·203									53	8	0·028	34	0·119	0·09
Total	3·40				29	0·987			9	0·316			25	0·857			7	0·227		2	0·071	28	0·942	0·85

TABLE 27. Extractions Performed at *p*H 5 and Room Temperature.

e_{7a} ε_{18B}	0·43	475	2·73	100	21	0·090	4·76	44	9	0·039	6·73	88	18	0·077	17·3	41	9	0·039				43	0·185	0·11
e_{6ab} ε_{18B}	0·55	484	3·04	125	26	0·143	4·39	52	10	0·055	6·37	125	26	0·143	17·4	48	10	0·055				28	0·154	0·14
e_{5abc} ε_{18B}	0·70	540	2·97	206	38	0·266					5·99	164	31	0·217	15·9	34	6	0·042				25	0·175	0·17
e_{4abcd} ε_{18B}	0·79	232	3·22	89	38	0·300					6·65	91	39	0·308	18·9	25	11	0·087				12	0·095	0·20
e_{3ab} ε_{18B}	0·36	374	*3·75*	112	30	0·108					6·99	118	32	0·115					12	3	0·011	35	0·126	0·09
e_{2ab} ε_{18B}	0·33	272					*3·96*	154	56	0·185	7·29	78	29	0·096								15	0·049	0·08
e_{1abc} ε_{18B}	0·35	658					4·03	383	58	0·203									53	8	0·028	34	0·119	0·09
Total	3·51				26	0·907			14	0·482			27	0·956			6	0·223		1	0·039	26	0·903	0·88

TABLE 28. Extractions Performed at *p*H 7 in the Cold Room (~ +2° to +4° C).

e_{7a} ε_{19A}	0·37	366	3·26	12	3	0·011					6·91	186	51	0·189					42	12	0·044	34	0·126	0·09
e_{6ab} ε_{19A}	0·37	389	3·34	4	1	0·004					6·71	158	41	0·152								58	0·214	0·09
e_{5abc} ε_{19A}	0·41	540	3·17	85	16	0·066					6·16	208	38	0·156	15·3	28	5	0·020				41	0·168	0·10
e_{4abc} ε_{19A}	1·03	500	2·87	218	44	0·453					5·87	38	8	0·082	15·2	33	6	0·062	101	20	0·206	22	0·227	0·26
e_{3ab} ε_{19A}	0·38	302	3·22	125	41	0·156					6·30	53	18	0·068					48	16	0·061	25	0·095	0·09
e_{2ab} ε_{19A}	0·19	318					*3·51*	153	48	0·091									94	30	0·057	22	0·042	0·05
e_{1abc} ε_{19A}	0·21	459					4·19	312	68	0·143												32	0·067	0·05
Total	2·96				23	0·690			8	0·234			22	0·647			3	0·082		12	0·368	32	0·939	0·73

TABLE 29. Extractions Performed at *p*H 5 in the Cold Room (~ +2° to +4° C).

e_{7a} ε_{19B}	0·24	233	3·32	16	7	0·017					7·02	74	32	0·077	18·5	15	6	0·014	120	52	0·125	3	0·007	0·06
e_{6ab} ε_{19B}	0·26	365	2·96	31	8	0·021		17	5	0·013	6·67	149	41	0·107	17·9	35	10	0·026				36	0·093	0·07
e_{5abc} ε_{19B}	0·56	445	2·88	121	27	0·151	4·15	30	7	0·039	6·30	161	36	0·202	15·0	38	9	0·050				21	0·118	0·14
e_{4abc} ε_{19B}	1·50	588	2·84	243	41	0·615	4·11	22	4	0·060	5·61	132	22	0·330	14·5	29	5	0·075				28	0·420	0·37
e_{3ab} ε_{19B}	0·46	515	3·10	177	34	0·157					6·27	176	34	0·156					49	10	0·046	22	0·101	0·12
e_{2ab} ε_{19B}	0·20	271					*3·78*	94	35	0·070	6·87	55	20	0·040					89	33	0·066	12	0·024	0·05
e_{1abc} ε_{19B}	0·21	459					4·19	312	68	0·143												32	0·067	0·05
Total	3·43				28	0·961			9	0·325			27	0·912			5	0·165		7	0·237	24	0·830	0·86

TABLE 30. Composition of Different Sub-fractions from $e_{3ab}\ \varepsilon_{19A}$ and $e_{3ab}\ \varepsilon_{19B}$.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram				Inhomogen. material not in diagram		Grams of protein per 100 ml. of serum
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.		
$(e_3\ \varepsilon_{19A})^7_3$	0·08	381	2·94	112	29	0·023	4·16	52	14	0·011					16·3	1						57	0·046		
$(e_3\ \varepsilon_{19A})^7_4$	0·04	233	3·10	86	37	0·015					7·04	37	16	0·006					21	9	0·004	38	0·015		
$(e_3\ \varepsilon_{19A})^5_4$	0·06	288	*3·50*	166	58	0·035									18·6	10	3	0·002	100	35	0·021	4	0·002		
$(e_3\ \varepsilon_{19A})_n$	0·04	210	*3·26*	83	40	0·016	4·22	33	16	0·006	6·06	15	7	0·003					7	3	0·001	34	0·014		
Total	0·22				40	0·089			8	0·017			4	0·009			1	0·002		12	0·026	35	0·077		
$(e_3\ \varepsilon_{19B})^7_2$	0·05	139	3·09	14	10	0·005					7·59	20	14	0·007								76	0·038		
$(e_3\ \varepsilon_{19B})^5_3$	0·06	288	3·07	59	20	0·012	4·37	16	6	0·003	7·04	66	23	0·014	18·5	8	3	0·002				48	0·029		
$(e_3\ \varepsilon_{19B})^7_3$	0·10	398	3·29	72	18	0·018	4·48	15	4	0·004	6·30	200	50	0·050					7	2	0·002	26	0·026		
$(e_3\ \varepsilon_{19B})^5_4$	0·07	343	*3·29*	168	49	0·034					5·65	74	22	0·016								29	0·020		
Total	0·28				25	0·069			2	0·007			31	0·087			1	0·002		1	0·002	40	0·113		

During these extraction experiments it was observed that the character of the precipitate changed. To begin with it had the same appearance as a serum albumin precipitate and could very easily be suspended in the extraction liquid. But during the extraction with '0·40 Am_2SO_4' at room temperature (ε_{18}) or with '0·37 Am_2SO_4' in the cold room (ε_{19}), the consistency changed and it became tough, sticky and difficult to suspend. Similar properties have often been observed, especially in precipitates obtained at '0·33' to '0·37 Am_2SO_4'.

The different fractions were investigated in the ultracentrifuge and the results are summarized in Tables 26—30. Some of the fractions seemed at first, according to the ultracentrifuge diagrams, to be comparatively pure with respect to fetuin. Two of these $e_3\,\varepsilon_{19A}$ and $e_3\,\varepsilon_{19B}$ were refractionated and the results are included in the tables.

On comparison of the tables, it is seen that the main part of the fetuin is extracted within somewhat narrower limits at the lower temperature, ε_{19}, than at room temperature. The tables also indicate a somewhat greater solubility of the fetuin at the lower temperature. The relative amounts of fetuin, 'albumin', globulin and the 20-component are almost the same in three of the preparations, but are lower in ε_{19A}, whereas the amount of inhomogeneous material is about 50 per cent higher in the latter case. The percentage of the 20-component is only about half that found in the three other preparations. The ratios fetuin/globulin and fetuin/albumin are very much the same in all four preparations. The hopes once placed in this method as a means of obtaining pure fetuin have not been fulfilled (*cf.* Pedersen, 1944).

c. Fœtal Serum from Cow.

The results obtained in the fractionation experiments with calf serum showed that fetuin was present in larger quantities in the new-born calf than at later stages. It therefore seemed reasonable to examine the blood from calf's fœtus with regard to its content of fetuin. From earlier investigations on calf and foal blood it is known that a very marked change takes place in the salt precipitability (Howe, 1921, b) and in the electrophoretic behaviour (Jameson *et al.*, 1942, San Clemente and Huddleson, 1943, and Polson, 1943) of the serum from a new-born animal after it has received colostrum. Immediately after the birth it is not possible to detect any measurable amount of Howe's euglobulin in the blood, but some hours after the animal has been

suckled for the first time considerable quantities of euglobulin may be found. The electrophoretic experiments showed similarly that γ-globulin could hardly be detected until some hours after the animal had been fed colostrum. The content of α-globulin is very high just after birth, but eventually decreases to its final value. The β-globulin starts at a rather low level but increases successively. In some recent experiments (Pedersen, 1944), it was found that 'normal globulin' (with $s_{20} \sim 7$) was present in very small amounts only, the great majority of the globulin fraction being fetuin. In the following account some fractionation experiments on serum from cow's fœtus and from a few other species will be described.

Fractionation Experiment No. 17. The serum used in this experiment was obtained from an eight to nine months old fœtus. The fractionation is described in some detail.

150 ml. of serum was diluted with 150 ml. 0·2 M-NaCl*. Addition of 150 ml. 'Am_2SO_4, *p*H 7' did not produce any precipitate. On adding a further 27 ml., $g_2^7\zeta_1$ was obtained. This was washed once with 100 ml. '0·42 Am_2SO_4, *p*H 7'. The *p*H of the mother liquor was changed to 5 by adding 2·7 ml. 1·0 N-H_2SO_4 and the deposit $g_2^5\zeta_1$ was obtained and was washed with 100 ml. '0·42 Am_2SO_4, *p*H 5'. The *p*H of the mother liquor was changed to 7 by adding 2·8 ml. 1·0 N-NaOH, and a further 24 ml. 'Am_2SO_4, *p*H 7' was added which procedure resulted in $g_3^7\zeta_1$. This was washed with 100 ml. '0·45 Am_2SO_4, *p*H 7'. The *p*H of the mother liquor (*p*H = 6·65) was adjusted to 5 by adding 3·0 ml. 1·0 N-H_2SO_4; the resulting precipitate $g_3^5\zeta_1$, was washed with 100 ml. '0.45 Am_2SO_4, *p*H 5'. The *p*H of the mother liquor (*p*H = 5·15) was again changed to 7 by adding 3·2 ml. 1·0 N-NaOH, and a further 45 ml. 'Am_2SO_4, *p*H 7' was added; this yielded $g_4^7\zeta_1$, which was washed with 100 ml. '0·50 Am_2SO_4, *p*H 7'. The *p*H of the mother liquor was brought back to 5 by adding 3·0 ml. 1·0 N-H_2SO_4, and the resulting precipitate, $g_4^5\zeta_1$, was washed with 100 ml. '0·50 Am_2SO_4, *p*H 5'. The *p*H of the mother liquor was returned to 7 by adding 3·1 ml. 1·0 N-NaOH, and a further 54 ml 'Am_2SO_4, *p*H 7' was added but did not give any precipitate. The addition of 3·9 ml. 1·0 N-H_2SO_4 to give *p*H 5 resulted in the precipitate $g_5^5\zeta_1$, which was washed with 100 ml. '0·55 Am_2SO_4, *p*H 5'. The *p*H of the mother liquor was again brought to 7 by adding 4·0 ml. 1 N-NaOH, and a further 150 ml. 'Am_2SO_4, *p*H 7' was added which gave $ga_2^7\zeta_1$. The precipitate was washed with 100 ml. '0·65 Am_2SO_4, *p*H 7'. By adding 3·0 ml. 1·0 N-H_2SO_4 to the mother liquor its *p*H was changed to 5·1 which resulted in a heavy precipitate, $ga_2^5\zeta_1$, which probably consisted mainly of albumin. It was washed with 100 ml. '0·65 Am_2SO_4, *p*H 5'. The addition of 1·6 ml. 1·0 N-H_2SO_4 to the mother liquor gave *p*H 4·7 but no precipitate. A further 2·2 ml. 1·0 N-H_2SO_4 resulted in *p*H 4·1, but produced only a slight turbidity. Finally, after the addition of solid ammonium sulphate, the precipitate $a_3\zeta_1$ was obtained. The amounts of protein in the individual fractions may be found in the second column of Table 31, which also summarizes the results from the ultracentrifugal examination of the different fractions.

A glance at Table 31 immediately shows the great difference between the serum from the calf and that from the fœtus In the latter case the fetuin predominates, and the ordinary globulin is present only in comparatively small amounts. It thus seems that it would be preferable to use the fœtal serum as the starting material in the preparation of fetuin.

An attempt was made to purify $g_3^5\zeta_1$ further by dialysis against 0·02 M-NaCl and adjustment of the *p*H of the protein solution to 5. The filtrate (96 per cent of the protein) gave almost the same result as that for the original solution (fetuin: 68 per cent, 20-component: 5 per cent and inhomogeneous material: 27 per cent), whereas the precipitate (4 per cent of the protein) was found to be very inhomogeneous with the approximate composition: 40 per cent fetuin and 60 per cent normal globulin.

Fractionation Experiment No. 18. To 250 ml. of the same serum as in the preceding experiment, 250 ml. 0·2 M-NaCl was added and the total globulin was precipitated in the usual manner with 500 ml. 'Am_2SO_4, *p*H 7'. The precipitate, $G\zeta_2$, was washed three times with 200 ml. '0·50 Am_2SO_4, *p*H 7'. The protein in the first wash-solution was precipitated and gave the solution $W_1G\zeta_2$. During the last washing one of the centrifuge tubes cracked and approximately 35 per cent of the precipitate was lost. In Table 32 the corresponding value for $G\zeta_2$ has been corrected for this loss.

The salt concentration of the mother liquor from $G\zeta_2$ was then increased to '0·55 Am_2SO_4', but neither at *p*H 7 nor at *p*H 5 did any precipitate appear. At '0·60 Am_2SO_4' the precipitate, $ga_2^5\zeta_2$, was not formed until just before the *p*H reached 5·10. It was washed with 200 ml. '0·65 Am_2SO_4, *p*H 5'. When the solution was further acidified to *p*H 4·7, $a_1\zeta_2$ was obtained. It was washed with 100 ml. '0·65 Am_2SO_4'. Changing the *p*H to 4·1 did not produce any deposit, but, after the addition of solid ammonium sulphate, $a_3\zeta_2$ was deposited. The results of the ultracentrifugal examination of the different fractions are summarized in Table 32.

Comparison of the total values for the different components in the Tables 31 and 32 shows that they agree within the experimental error. The slight difference may very well be explained as due to the amount of protein discarded together with the second and third wash-solutions. If the total globulin values are compared, there is a slight difference in so far as no globulin was noticed in $G\zeta_2$, but the amount of 'inhomogeneous material not observed in the sedimentation diagram' was 5 per cent higher than in ζ_1.

Part of the $G\zeta_2$, after dialysis against 0·2 M-NaCl*, was diluted with 0·2 M-NaCl* to give 200 ml. of a one per cent globulin solution. Addition of both 100 ml. 'Am_2SO_4, *p*H 7' and a further 18 ml. of the same solution did not produce any pre-

TABLE 31. COMPOSITION OF DIFFERENT FRACTIONS FROM FŒTAL COW SERUM (FRACTIONATION EXPERIMENT No. 17).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$g_2^7\,\zeta_1$	0·09	201	3·05	153	74	0·067					5·6	40	19	0·017		15	7	0·006						0·06
$g_2^5\,\zeta_1$	0·66	440	2·56	364	83	0·548					6·82	9	2	0·013	16·4	24	5	0·033				10	0·066	0·44
$g_3^7\,\zeta_1$	0·19	532	2·51	304	57	0·108					8·05	78	15	0·029	15·1	42	8	0·015				20	0·038	0·12
$g_3^5\,\zeta_1$	0·48	644	2·50	442	69	0·331									14·4	37	6	0·029				25	0·120	0·32
$g_4^7\,\zeta_1$	0·07	287	3·06	168	59	0·041						14	5	0·004	17·4	12	4	0·003				32	0·022	0·05
$g_4^5\,\zeta_1$	0·10	279	3·15	124	44	0·044					5·19	14	5	0·005								51	0·051	0·07
$g_5^5\,\zeta_1$	0·13	382	*3·24*	232	61	0·079					7·24	52	13	0·017								26	0·034	0·09
$ga_2^7\,\zeta_1$	0·11	264	*3·47*	172	65	0·072																35	0·038	0·07
$ga_2^5\,\zeta_1$	2·26	629					3·82	474	75	1·695												25	0·565	1·51
$a_3\,\zeta_1$	0·37	366					4·23	240	66	0·244												34	0·126	0·25
'Total globulin'	1·72				71	1·218							5	0·085			5	0·086				19	0·331	1·15
Total	4·46				29	1·290			43	1·939			2	0·085			2	0·086				24	1·060	2·98

TABLE 32. COMPOSITION OF DIFFERENT FRACTIONS FROM FOETAL COW SERUM (FRACTIONATION EXPERIMENT No. 18).

$G\,\zeta_2$	3·23	403	2·79	290	72	2·326									15·2	18	4	0·129				24	0·775	1·29
$W_1G\,\zeta_2$	0·27	358					3·97	230	64	0·173									31	9	0·024	27	0·073	0·11
$ga_2^5\,\zeta_2$	2·44	573					3·87	436	76	1·854												24	0·586	0·98
$a_1\,\zeta_2$	1·24	605					3·79	501	83	1·029												17	0·211	0·50
$a_3\,\zeta_2$	0·42	351					4·49	257	73	0·307												27	0·113	0·17
Total	7·33				32	2·326			43	3·190							2	0·129			0·024	23	1·685	3·05

TABLE 33. COMPOSITION OF THE SUB-FRACTIONS FROM $G\zeta_2$.

$g_2^5\zeta_2$	1·82	473	2·60	395	84	1·529									14·5	16	3	0·055				13	0·236	0·73
$g_3^5\zeta_2$	0·55	549	2·58	346	63	0·347									13·8	32	6	0·033				31	0·170	0·22
$g_4^7\zeta_2$	0·14	279	3·04	200	72	0·101					7·65	32	11	0·015								17	0·024	0·06
$g_5^7\zeta_2$	0·11	201	3·17	150	75	0·083	4·86	49	24	0·026					15·2	1	1	0·001						0·04
'Total globulin'	2·62				79	2·060			1	0·026			1	0·015			3	0·089				16	0·430	1·05

TABLE 34. COMPOSITION OF DIFFERENT FRACTIONS FROM FŒTAL COW SERUM (FRACTIONATION EXPERIMENT NO. 19).

$g_3^7\zeta_3$	0·62	538	2·56	297	55	0·341									13·6	78	15	0·093				30	0·186	0·31
$g_3^5\zeta_3$	0·92	530	2·47	368	70	0·644									13·8	34	6	0·055				24	0·221	0·46
$g_4^7\zeta_3$	0·15	311	3·03	118	38	0·057					7·32	21	7	0·010	17·8	18	6	0·009	87	28	0·042	21	0·032	0·07
$g_4^5\zeta_3$	0·23	373	2·87	248	67	0·154									15·9	8	2	0·005	39	10	0·023	21	0 048	0·12
$g_5^5\zeta_3$	0·19	539	2·96	245	45	0·086		68	13	0·025	7·31	36	7	0·013	16·3	27	5	0·009	51	9	0·017	21	0·040	0·09
$ga_1^5\zeta_3$	0·09	264	*3·47*	141	54	0·049									18·7	8	3	0·003	72	27	0·024	16	0·014	0·05
$ga_2^7\zeta_3$	0·08	193	*3·49*	84	44	0·035					8·3	29	15	0·012	*12·6*	20	10	0·008	36	19	0·015	12	0·010	0·04
$ga_2^5\zeta_3$	1·39	514					3·90	398	77	1·070												23	0·320	0·69
$a_2\zeta_3$	1·61	413					4·35	303	73	1·175												27	0·435	0·81
$a_3\zeta_3$	0·65	540					*3·17*	69	13	0·085												31	0·201	0·32
							4·31	301	56	0·364														
$g_3^7\zeta_3$ [1]		436	2·66	268	62										14·9	37	8					30		
'Total globulin'	2·11				61	1·282			1	0·025			1	0·023			8	0·171		4	0·082	25	0·527	1·05
Total	5·93				23	1·366			46	2·719			1	0·035			3	0·182		2	0·121	25	1·507	2·96

[1] After being spun in the air-driven centrifuge at 27,000 R.P.M. for 6 hours.

cipitate. After acidification to *p*H 5, $g_2^5\zeta_2$ was obtained; it was washed with 100 ml. '0·42 Am_2SO_4, *p*H 5'. Neutralization of the mother liquor (*p*H 5·34) with 2·1 ml. 1 N-NaOH and addition of 16 ml. 'Am_2SO_4, *p*H 7' did not yield any precipitate. At *p*H 5 $g_3^5\zeta_2$ was formed. It was washed with 100 ml. '0·45 Am_2SO_4, *p*H 5'. After neutralization and addition of 30 ml. 'Am_2SO_4, *p*H 7' $g_4^7\zeta_2$ was obtained and was washed with 50 ml. '0·50 Am_2SO_4, *p*H 7'. No precipitation occurred after acidification to *p*H 5, but after neutralization and addition of a further 36 ml. 'Am_2SO_4, *p*H 7', $g_5^7\zeta_2$ was formed. This was washed with 75 ml. '0·55 Am_2SO_4, *p*H 7'. No precipitate appeared after acidification to *p*H 5, and the addition of solid ammonium sulphate only gave rise to a slight turbidity. The results from the ultracentrifugal investigation of the different fractions have been summarized in Table 33.

It is seen from the table that a definite improvement in the purification of the fetuin is attained through the re-fractionation. Even the relative fetuin concentration in 'total globulin' is higher than in $G\zeta_2$, and if $g_2^5\zeta_2$ alone is taken into account the improvement is still more pronounced.

Attempts were made to crystallize the fetuin from $g_2^5\zeta_2$. The *p*H of the solution was adjusted to 5 and the liquid was transferred to a cellophane bag which was then rotated in a '0·40 Am_2SO_4, *p*H 5' solution at room temperature. The salt solution was renewed twice at intervals of 4 to 5 hours. The precipitate collected at the bottom of the bag as an almost transparent gel. It was left for some days, but no crystalls were formed. Later the solution was allowed to stand in the refrigerator at + 2° C, but the result remained the same. The precipitate, 83 per cent of the protein, was dissolved in 0·2 M-NaCl* and dialysed first against a similar solution and then against two phosphate buffers for electrophoresis experiments. According to these the material was electrophoretically homogeneous. The two solutions were also run in the ultracentrifuge with the following result. The solution at *p*H 7·5, fetuin: 63 per cent, 20-component: 2 per cent and inhomogeneous material not in the sedimentation diagram: 35 per cent. For the solution at *p*H 5·7 the corresponding values were 78, 2 and 20 per cent, respectively. There was thus no improvement as compared with $g_2^5\zeta_2$, but more inhomogeneous material was found. The protein in the mother liquor, 17 per cent of the total used for the experiment, was precipitated by addition of solid ammonium sulphate. The values for fetuin, 20-component and inhomogeneous material were 69, 10 and 21 per cent, respectively. The 20-component had thus been increased in this fraction.

Fractionation Experiment No. 19. The starting material in this case was a serum similar to that used in Nos. 17 and 18, but from a different individual. The procedure was almost the same as in Experiment No. 17 except at the very beginning; the washings, however, were now made twice with 100 ml. each time.

200 ml. of serum was mixed with the same volume of 0·2 M-NaCl* and of 'Am_2SO_4, *p*H 7', but no precipitate occurred either at this concentration or after a further addition of 36 ml. Am_2SO_4. When the salt concentration was increased to '0·40 Am_2SO_4, *p*H 7' the first deposit occurred. It was washed twice with 100 ml. '0·45 Am_2SO_4, *p*H 7'. When the mother liquor was acidified to *p*H 5, $g_3^5\zeta_3$ was precipitated. The remainder of the fractionation followed the procedure in Experiment No. 17. When the *p*H of the mother liquor from $ga_2^5\zeta_3$ was determined, it was found to be 4·7 instead of 5·1. This fraction was thus undoubtedly a mixture corresponding to both ga_2^5 and a_1. The results of the ultracentrifugal examination of the different fractions have been summarized in Table 34.

If Tables 31 and 34 are compared, it is seen that the last serum contains less fetuin than that used in Exp. No. 17. This is still more pronounced when the values for total globulin are alone taken into account.

Fractionation Experiment No. 20. Since the preceding experiments had shown that the fœtal sera contained very little or no normal globulin, a new way for preparing fetuin was tried on the serum used in Experiment No. 19.

To 150 ml. of fœtal serum were added first 150 ml. 0·2 M-NaCl* and then 300 ml. saturated Am_2SO_4. The precipitate $G_{\text{I}}\zeta_4$ was washed twice with 200 ml. '0·50 Am_2SO_4' and finally dissolved in 25 ml. water (volume of solution 45 ml.). For examination 4·5 ml. was removed and dialysed against 0·2 M-NaCl*, while the remainder was diluted with 277·5 ml. water and mixed with 300 ml. 'Am_2SO_4'. The precipitate, $G_{\text{II}}\zeta_4$, was washed with 200 ml. '0·50 Am_2SO_4' and dissolved in 25 ml. water (Volume: 56 ml.). For further examination 5·6 ml. was removed, as before. The rest was treated as previously described and yielded $G_{\text{III}}\zeta_4$. After dialysis against 0·2 M-NaCl*, the whole solution was spun for 6 hours in the air-driven centrifuge at 27,000 R.P.M. Each tube was filled with 6 ml. solution and the upper 5·5 ml. was removed as $G_{\text{IIIA}}\zeta_4$. The concentration of this latter solution was 80 per cent of that in $G_{\text{III}}\zeta_4$. A small portion of $G_{\text{IIIA}}\zeta_4$ was spun once more for the same length of time and gave $G_{\text{IIIB}}\zeta_4$. The solutions, except $G_{\text{III}}\zeta_4$, were examined in the ultracentrifuge. Globulin could not be detected in any of these. In the wash-solution from $G_{\text{I}}\zeta_4$, the main peak was of a composite nature and could best be explained as a mixture of albumin and fetuin. Table 35 gives the relative amount in per cent of the various components at the different stages of the purification of fetuin.

Table 35.

Fraction	Fetuin	Albumin	Globulin	20-component	Inhomogeneous
$G_I\zeta_4$	58	0	0	9	33
$WG_I\zeta_4$	61		0	2	37
$G_{II}\zeta_4$	64	0	0	8	28
$G_{IIIA}\zeta_4$	72	0	0	3	25
$G_{IIIB}\zeta_4$	80	0	0	2	18

From the table it is seen that the relative concentration of the fetuin gradually increases, while those of the 20-component and of the inhomogeneous material decrease. The greatest improvements take place during the high-speed centrifugation. By choosing the salt concentration for the re-fractionation as '0·40 Am_2SO_4' instead of '0·50 Am_2SO_4' a somewhat better result might perhaps be achieved.

B. Human Serum and Plasma.

a. Normal Adult Human Serum and Plasma.

The 'total globulin' fractions from human sera were studied by McFarlane (1935, e), who found that they were mixtures of globulin and, according to his view, albumin. Some time ago Kekwick (1940) prepared a normal human γ-globulin, which was homogeneous both in sedimentation and in electrophoresis. This globulin was precipitated from serum by the addition of Na_2SO_4 to 13 g./100 ml.

The fractionation of human serum and plasma was attempted at an early date in this investigation, but, as the fractions obtained were all mixtures containing a smaller or greater number of components, further work on these liquids was postponed until more informations had been gained from experiments with the more readily available cow serum. Some of the observations made during the first period may be of a general interest, however, and will be mentioned briefly together with a few recent fractionation experiments.

Fractionation Experiment No. 21. In the first experiment human plasma (KOP $^{23}/_4$ 42) was diluted with an equal volume of a citrate-phosphate buffer with *p*H about 8. Precipitates containing fibrinogen were obtained at '0·19', '0·26' and '0·29 Am_2SO_4'. The deposits were not washed, but were sedimented tightly down in

the centrifuge tubes, and the mother liquor adhering to the walls of the tubes was allowed several minutes to drain away. Two thirds of the first fraction consisted, according to the sedimentation diagrams, of a component with $s_{20} \sim 8$, while the remainder was albumin. The unexpectedly high albumin content was confirmed in an electrophoresis experiment (albumin: 31 per cent, fibrinogen 10 per cent and γ-globulin 59 per cent). The high value, $s_{20} \sim 8$, accords with the sedimentation constant found by Holmberg (1944, a) for purified fibrinogen, but as, according to electrophoresis, the 'globulin fraction' contained six times as much γ-globulin as fibrinogen, the high value for s_{20} seems a little curious. Globulin sedimentation constants of the same order have been observed from time to time in the fraction obtained in the range '0·4 to 0·5 Am_2SO_4', but the total globulin content in the serum is comparatively low. The presence of this γ-globulin and of the albumin in the fibrinogen fraction would seem to indicate that these two proteins are in some way specifically precipitated together with the fibrinogen or adsorbed on the deposited particles; the mere adhesion of the mother liquor can in no way be responsible for such large amounts of 'foreign' proteins.

All the fractions from '0·26' to '0·45 Am_2SO_4' were found, both in sedimentation and electrophoresis, to contain about 10 per cent of albumin. This emphasizes the necessity of washing the precipitates.

The second albumin fraction, obtained at '0·65—0·70 Am_2SO_4, pH 4·7', which appeared homogeneous in the ultracentrifuge, was found to contain 5 per cent α- and 9 per cent β-globulin. The last two components were found in the largest amounts in the fractions precipitated between '0·45' and '0·60 Am_2SO_4'.

Fractionation Experiment No. 22. In the next experiment, which was performed shortly after that just described, serum (FC $^{23}/_{4}$ 42) was diluted with an equal volume of a phosphate buffer with pH = 6·0 and fractions were taken at the concentrations '0·33', '0·375', '0·41', '0·46', '0·50' and '0·60 Am_2SO_4'; a single albumin fraction was finally obtained by acidification to pH 4·75.

The precipitates were not washed and also contained amounts of albumin similar to those found in the preceding experiment. They were dissolved in phosphate buffers with $\mu = 0{\cdot}01$ and pH = 6 and dialysed against the same solution. The deposits from the dialysis were dissolved in 0·2 M-NaCl. All the fractions contained at least two components besides the albumin.

The more soluble fraction from '0·46 Am_2SO_4' was found to be very rich in the 20-component (30—35 per cent). Electrophoretic examination of the same solution gave 44 per cent of α-globulin (albumin 17, β_1-14, β_2-19 and γ-globulin 6 per cent), a strong indication that the 20-component is an α-globulin, but not *vice versa*, as several solutions containing no 20-component, according to electrophoresis, contained α-globulin.

It has always been found that the precipitates from human sera and plasma in the range '0·50' to '0·60 Am_2SO_4' are very difficult to centrifuge clear. The precipitates generally rise to the top of the centrifuge tubes, and it is necessary to filter the solutions in order to collect the fraction, which corresponds well with the method for isolating the labile β-component from serum described on p. 30. Electrophoretic examination of these fractions showed that they were rich in β_1- and β_2-globulins, but in addition both albumin and α-globulin were always present.

TABLE 36. COMPOSITION OF DIFFERENT FRACTIONS FROM HUMAN SERUM (FRACTIONATION EXPERIMENT NO. 23).

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material					Grams of protein per 100 ml. of serum
																			in sedimentation diagram			not in sedimentation diagram		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$g_1^7\ \alpha_{11}$	1·80	281									6·51	184	65	1·170								35	0·630	1·80
$g_2^7\ \alpha_{11}$	0·28	330					4·10	24	7	0·020	6·41	210	64	0·179	18·7	19	6	0·017				23	0·064	0·28
$g_3^7\ \alpha_{11}$	0·18	311	3·29	21	7	0·013					6·81	151	49	0·088	18·4	45	14	0·025				30	0·054	0·18
$g_3^5\ \alpha_{11}$	0·30	358	2·64	18	5	0·015	4·16	22	6	0·018	5·84	57	16	0·048	17·3	72	20	0·060	69	19	0·057	34	0·102	0·30
$g_{4s}^7\ \alpha_{11}$	0·12	123					4·08	15	12	0·014	6·66	55	45	0·054	18·2	34	28	0·034				15	0·018	0·12
$g_{4p}^7\ \alpha_{11}$	0·06	77																		50	0·030	50	0·030	0·06
$g_5^5\ \alpha_{11}$	0·22	287					4·26	145	51	0·112	7·02	30	10	0·022	19·2	10	4	0·009	38	13	0·029	22	0·048	0·22
$ga_1^5\ \alpha_{11}$	1·33	402					4·03	299	75	0·997									25	6	0·080	19	0·253	1·33
$ga_2^5\ \alpha_{11}$	1·12	302					4·02	267	88	0·986												12	0·134	1·12
$a_1\ \alpha_{11}$	0·31	217					4·17	166	76	0·236												24	0·074	0·31
'Total globulin'	2·96				1	0·028			5	0·164			53	1·561			5	0·145		4	0·116	32	0·946	2·96
Total	5·72					0·028			42	2·383			27	1·561			3	0·145		3	0·196	25	1·407	5·72

TABLE 37. COMPOSITION OF DIFFERENT FRACTIONS FROM HUMAN SERUM (FRACTIONATION EXPERIMENT No. 24).

$eug_1\ \alpha_{12}$	0·36	241									6·98	36	15	0·054					57	24	0·086	61	0·220	0·36
$g_1^7\ \alpha_{12}$	0·53	439	3·33	41	9	0·048					6·30	261	59	0·313	16·8	7	2	0·010				30	0·159	0·53
$g_2^7\ \alpha_{12}$	0·33	596		50	8	0·026					5·98	300	50	0·165	17·4	21	4	0·013				38	0·126	0·33
$g_3^7\ \alpha_{12}$	0·21	477									6·19	206	43	0·090	15·0	37	8	0·017				49	0·103	0·21
$g_4^7\ \alpha_{12}$	0·21	531	3·43	35	7	0·015	4·15	15	3	0·006	6·22	155	29	0·061	15·9	72	13	0·027				48	0·101	0·21
$g_5^7\ \alpha_{12}$	0·12	209	2·79	39	19	0·023					6·96	64	31	0·037	19·9	11	5	0·006				45	0·054	0·12
$ga_1^7\ \alpha_{12}$	0·22	170					4·55	114	67	0·147												33	0·073	0·22
$ga_2^7\ \alpha_{12}$	0·16	271					4·23	180	66	0·106					22	5	2	0·003	61	23	0·037	9	0·014	0·16
$ga_2^5\ \alpha_{12}$	0·95	582					3·81	426	73	0·694												27	0·256	0·95
$a_2\ \alpha_{12}$	0·39	124					4·40	126	100	0·390														0·39
$a_2^1\ \alpha_{12}$	0·49	492					3·95	344	70	0·343												30	0·147	0·49
'Total globulin'	1·76				7	0·112				0·006			41	0·720			4	0·073		5	0·086	43	0·763	1·76
Total	3·97				3	0·112			42	1·686			18	0·720			2	0·076		3	0·123	32	1·253	3·97

TABLE 38. COMPOSITION OF DIFFERENT FRACTIONS FROM HUMAN UMBILICAL SERUM.

	mg.				mg.				mg.				mg.				mg.			mg.		
$g_1^7\ \gamma_1$	106	358	3	6	2	2·1					6·57	216	60	63·6								38	40·3	1·06
$g_2^7\ \gamma_1$	28	186	3·30	8	4	1·1					6·85	117	63	17·6	20·8	11	6	1·7				27	7·6	0·28
$g_4^5\ \gamma_1$	46	342					*3·62*	43	12	5·5	7·00	116	34	15·7	17·8	109	32	14·7				22	10·1	0·46
$ga_2^7\ \gamma_1$	4	54	3·25	2	4	0·16	4·59	6	11	0·44	6·68	8	15	0·60	21·2	4	7	0·28	7	13	0·52	50	2·0	0·04
$a_1\ \gamma_1$	165	350					4·16	280	80	132·0												20	33·0	1·65
$a_2\ \gamma_1$	136	194					4·53	132	68	92·5												32	43·5	1·36
'Total globulin'	180				2	3·2			3	5·5			54	96·9			9	16·4				32	58·0	1·80
Total	485				1	3·36			48	230·44			20	97·5			3	16·68			0·52	28	136·5	4·85

Some experiments were carried out where the serum, before precipitation with ammonium sulphate, was dialysed against dilute salt solutions or water. None of the different fractions obtained was, however, of a tolerable purity and some were quite inhomogeneous mixtures. It was observed that when the *p*H of the serum was kept above 6 (perhaps just above *p*H 5·8) during the dialysis, the deposits formed were mixtures of comparatively homogeneous components. But when the sera were acidified to a *p*H below 5·5, the precipitates produced gave sedimentation diagrams showing more or less completely inhomogeneous material.

In this connection it may be of interest to note that Theorell (1930) found that the plasma-cholesterol in the *p*H range 5·5 to 6·0 is very easy to extract. He showed that this was due to the fact that cholesterol is bound in some way to fibrinogen and euglobulin above and below this *p*H value, but that the fibrinogen-cholesterol complex dissociates at about *p*H 6, while the euglobulin-cholesterol complex becomes unstable in the *p*H range 5·5 to 6·0. The albumin would only bind the cholesterol on the acid side of this range and it seems that the lipid-protein complexes in plasma are more stable on the acid side of *p*H 5·5 than on the alkaline side.

From what has been said it appears that the old method for preparing serum globulin, viz. by dilution with water and acidification with gaseous CO_2, would be a fairly gentle method. This also appears to be true in so far as rather little inhomogeneous material is produced in either the euglobulin or the globulin precipitated later with, for example, ammonium sulphate. The globulins obtained are, however, by no means pure; all fractions are mixtures of albumin (or 'X-protein'), globulin and the 20-component.

Fractionation Experiment No. 23. In this experiment 100 ml. of normal human serum (IP $^{18}/_{9}$ 44) was diluted with 100 ml. 0·2 M-NaCl and fractions were taken (or it was attempted to take them, but without success) at the following concentrations and *p*H values: '0·33, *p*H 7', '0·37, *p*H 7', ('0·37, *p*H 5'), '0·40, *p*H 7', '0·40, *p*H 5', '0·45, *p*H 7', ('0·45, *p*H 5'), ('0·50, *p*H 7'), '0·50, *p*H 5', ('0·55, *p*H 7'), '0·55, *p*H 5', ('0·60, *p*H 7'), '0·60, *p*H 5', '0·60, *p*H 4·7', ('0·60, *p*H 4·1'); solid ammonium sulphate was finally added, but no precipitate was found; the solution merely became a little turbid. The precipitate at '0·50 Am_2SO_4, *p*H 5', $g_3^5\,\alpha_{11}$, could not be sedimented down, and it was therefore removed from the solution by filtration. The precipitates were all washed with 100 ml. of solutions more concentrated by '0·05 Am_2SO_4' than the mother liquors and with the proper *p*H. The results of the ultracentrifugal examination of the different fractions are given in Table 36.

By the dialysis of $g_4^7\,\alpha_{11}$ against 0·2 M-NaCl*, some precipitate was formed. The soluble fraction has been marked $g_{4s}^7\,\alpha_{11}$, while the precipitate, after dissolution in 0·1 M-Na_2CO_3 and dialysis against 0·2 M-NaCl, has been called $g_{4p}^7\,\alpha_{11}$.

From the table it is seen that only the first and the two last fractions from α_{11} give sedimentation diagrams with a single homogeneous component.

Fractionation Experiment No. 24. In this experiment 100 ml. of another normal human (KOP $^{13}/_{0}$ 44) serum was adjusted to *p*H 5·5 and dialysed against 0·01 M-NaCl*, whereby $eug_1\,\alpha_{12}$ was formed. The filtrate was diluted with water and 2 M-NaCl* to give 200 ml. of a 0.2 M-NaCl*-serum solution. This was fractionated and the precipitates washed in exactly the same manner as in the preceding experiment, but there was some slight difference as to where some of the precipitates were obtained.

Although the two sera fractionated had about the same protein content, 7·20 and 7·32 g. per 100 ml. for α_{12} and α_{11} respectively, the sum of the protein contents of their different fractions were 3·97 and 5·72 respectively. The loss was thus greatest in the dialysed solution (α_{12}), but it seems that it occurred during the precipitation with ammonium sulphate, since the filtrate from the dialysis still contained 6·70 g. protein. It has often been found that the total yield of protein is less in cases where the serum has been dialysed against water or dilute salt solutions before the precipitation with ammonium sulphate.

The results from the ultracentrifugal examination of the different fractions are given in Table 37, p. 119.

The total amount of the globulin fraction is higher in α_{11} than in α_{12} as is also the case with the two sera, but α_{12} contains relatively more inhomogeneous material and less 'globulin' than α_{11}. It is seen from the table that the larger part of the $eug_1\,\alpha_{12}$ consists of inhomogeneous material, an indication that a solution at *p*H 5·5 is definitely too acid for producing the euglobulin. The rest of the globulin fraction also contains relatively more inhomogeneous material than the corresponding fractions from α_{11}, the other differences probably being due to individual differences between the two sera.

b. Pathological Human Serum.

From the foregoing results it is evident that the preparation of the pure normal human serum proteins, with the exception of a normal γ-globulin with $s_{20} \sim 7$, must undoubtedly be quite an intricate affair. In the case of some of the pathological serum proteins, however, the situation seems to be somewhat different, as the relative concentration of the pathological component may be comparatively high, which makes the isolation much easier.

In a few instances protein crystals have developed in such sera on standing in a cold room (Bonsdorff *et al.* (1937, 1938), Packalén (1939), Holmberg and Grönwall (1942), Holmberg (1944, a) and perhaps Bing (1940)). In some cases (Bonsdorff *et al.* and Bing, *l. c.*) two strata were formed in the serum, the lower one being a rather concentrated solution of the pathological globulin. Under such circumstances, the purification of these proteins is considerably facilitated, but the amount of serum available has usually been rather too small for further fractionation and purification.

McFarlane (1935, e) has studied fractions from several pathological sera. In some cases he obtained fairly uniform globulin fractions, while in others both the globulin and the albumin fractions were mixtures of two or more components.

Some myelomatosis globulins have been prepared by Kekwick (1940), three were found to show a normal globulin sedimentation in the centrifuge, but in one of these cases an electrophoresis experiment revealed the presence of a faster migrating component besides the γ-globulin. From a fourth myeloma serum an electrophoretically homogeneous β-globulin was prepared. In the centrifuge, however, this fraction was far from homogeneous, showing four distinct components with $s_{20} = 6{\cdot}54$, 9·26, 11·3 and 13·3. In the precipitation of this protein, Kekwick observed that the material thrown out of the solution with 20 g./100 ml. of Na_2SO_4 rose to the surface and had a sticky consistency. A similar behaviour is generally found with the *ga*-fractions from human sera.

From time to time comparatively large quantities of human serum and plasma (several hundred ml.) from persons with hyperglobulinemia have been put at the disposal of the author by Dr. Jan Waldenström, Upsala. The patients in question were treated several times at the University Medical Clinic with venesections and transfusions with normal blood (Waldenström, 1944, a). As the sera contained very large amounts of pathological globulins with $s_{20} \sim 20$ (see Fig. 17, p. 39), it was tempting to attempt the purification of these proteins. However, as the pathological sera have been regarded as being outside the scope of this paper, only the points of more general interest will be noted.

Fractionation Experiment No. 25. The serum used in this experiment was from Case II described in Waldenström, *l. c.* It was diluted with an equal volume of 0·2 M. phosphate buffer of pH = 7·5 and precipitated at '0·33 Am_2SO_4'. The pre-

cipitate came down as long threads of a jelly-like consistency, but settled rather soon to the bottom of the vessel. It was very difficult to pack by centrifuging and the total volume of the precipitate was as much as 80 ml. (from 250 ml. serum). The remainder of the heavy component came down at '0·375 Am_2SO_4' and had a volume of about 15 ml. The first precipitate, $g_1\beta_1$, was reprecipitated first at '0·32 Am_2SO_4, and then at '0·30 Am_2SO_4'. During the precipitation and the stirring of the solution $g_{1aA}\beta_1$ took up some air-bubbles and was very difficult to centrifuge down. It was again dissolved in water, reprecipitated at '0·35 Am_2SO_4', dissolved in water and called $g_{1aA'}\beta_1$. This solution was refractionated at '0·25', '0·28', '0·30', '0·31', '0·33', and about '0·45 Am_2SO_4'. The larger part of the precipitate, $g_{1aA'_3}\beta_1$, (volume of wet precipitate: 40 ml.) came down at '0·30 Am_2SO_4'. Ultracentrifugal examination at the different stages of purification showed that $g_1\beta_1$ contained considerable amounts of material sedimenting more slowly than the 20-component, but even after five precipitations $g_{1aA'_3}\beta_1$ still included about one fifth of slower sedimenting material. A new method of fractionation was then tried. One volume of $g_{1aA'_3}\beta_1$, dissolved in and dialysed against 0·2 M-NaCl, was diluted with fifty volumes of water. The euglobulin deposited, $(g_{1aA'_3}\beta_1)_1$, was centrifuged down and dissolved in 0·2 M-NaCl. It showed 3 components in the sedimentation diagram ($s_{20} = 19{\cdot}7$ +++, 25·0 +++ and 29·3 ++ at $\Delta n = 240$), no slower sedimenting material was observed. The pseudoglobulin remaining in solution was precipitated with ammonium sulphate $(g_{1aA'_3}\beta_1)_2$. In the centrifuge it also gave three components ($s_{20} = 7{\cdot}7+$, 17·6 ++++++, 24·4 + by $\Delta n = 290$).

The last result obtained indicated that methods other than precipitation with ammonium sulphate might lead to a more rapid purification of the 20-component. Different attempts were therefore made to prepare pure euglobulin from the serum.

Fractionation Experiment No. 26. Firstly a fractionation was tried by taking the successive deposits formed during a stepwise increase in the dilution of the serum with water. The precipitates at 1 : 20 and 1 : 100 consisted largely of the 20-component contaminated with both lighter and heavier components. The same was the case in other dilution experiments, and when the serum had been dialysed against water before dilution with water or with 0·001 M-NaH_2PO_4. When the euglobulin, obtained in the last case, was re-precipitated by dilution with a similar phosphate solution, the quantity of lighter components was about halved.

A still better result was obtained by a single dilution of the serum with 60 volumes Na_2HPO_4 with $\mu = 0{\cdot}001$. Re-precipitation with similar phosphate solutions did not seem to improve the purity of the 20-component to any great extent. The final purification of the euglobulin was made by fractional sedimentation in the high-speed air-driven centrifuge.

With the same serum and that from Case I of Waldenström (*l. c.*) a further series of purification experiments were carried out. Thus the sera were adjusted to *p*H 6 and fractionated by dialysis against solutions with decreasing ionic strength. In Case I the main part of the euglobulin was precipitated at $\mu = 0{\cdot}01$, while in Case II this already occurred at $\mu = 0{\cdot}02$. As was to be expected these voluminous precipitates

TABLE 39. COMPOSITION OF DIFFERENT FRACTIONS FROM RABBIT SERUM.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram				Inhomogen. material not in sedimentation diagram		Grams of protein per 100 ml. of serum
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	%	g.	
$g_1\,\mu_1$	0·176	196					4·61	5	3	0·005	7·07	141	72	0·127	20·8	5	2	0·004				23	0·040		0·35
$g_2\,\mu_1$	0·089	468					*3·92*	32	7	0·006	6·26	288	61	0·054								32	0·029		0·18
$g_3\,\mu_1$	0·050	272	3·28	29	10	0·005	4·46	14	5	0·003	6·60	146	54	0·027	17·7	13	5	0·002				26	0·013		0·10
$g_4\,\mu_1$	0·075	397	3·0	65	16	0·012	4·5	50	13	0·010	6·49	119	30	0·022	16·8	35	9	0·007				32	0·024		0·15
$g_5\,\mu_1$	0·049	389	2·84	34	9	0·004	4·22	98	25	0·012	6·54	44	11	0·006	17·4	103	26	0·013	18	5	0·002	24	0·012		0·10
$ga_1\,\mu_1$	0·110	595					3·84	297	50	0·055	6·36	45	8	0·009	15·2	139	23	0·025				19	0·021		0·22
$ga_2\,\mu_1$	0·129	468					3·97	352	75	0·097						3	1	0·001				24	0·031		0·26
$a_1\,\mu_1$	1·037	461					3·93	358	78	0·809									34	7	0·072	15	0·156		2·07
'Total globulin'	0·439				5	0·021			8	0·036			54	0·236			6	0·026			0·002	27	0·118		0·88
Total	1·715				1	0·021			58	0·997			14	0·245			3	0·052		5	0·074	19	0·326		3·43

TABLE 40. COMPOSITION OF DIFFERENT FRACTIONS FROM FŒTAL RABBIT SERUM.

	mg.					mg.				mg.				mg.				mg.				mg.		
$g_2\,\nu_1$	25	124									6·90	57	46	11·5	21·8	4	3	0·8	20	16	4·0	35	8·7		0·50
$g_4\,\nu_1$	11	108	3·24	19	18	2·0					7·13	25	23	2·5								59	6·5		0·22
$ga_1\,\nu_1$	24	162	2·81	10	6	1·4	4·37	100	62	14·9					19·7	20	12	2·9				20	4·8		0·48
$a_1\,\nu_1$	109	155					4·07	59	38	41·0												62	68·0		2·18
'Total globulin'	36				6	2·0							39	14·0			2	0·8		11	4·0	42	15·2		0·72
Total	169				2	3·4			33	55·9			8	14·0			2	3·7		3	4·0	52	88·0		3·38

contained considerable quantities of the other serum components. The further purification of the 20-component from the lighter and heavier contaminations was carried out in the manner just described.

c. Serum from the Umbilical Cord.

Fractionation Experiment No. 27. The serum used in this experiment was obtained from the umbilical blood of a new-born infant[1]. 10 ml. of serum was diluted with the same amount of 0.2 M-NaCl* and mixed with 10 ml. 'Am_2SO_4, *p*H 7'. Contrary to the behaviour of the other fœtal sera, a heavy precipitate, $g_1^7\gamma_1$ came down and was washed twice with 25 ml. '0·40 Am_2SO_4, *p*H 7'. To the mother liquor 1·8 ml. 'Am_2SO_4, *p*H 7' was added, and the precipitate $g_2^7\gamma_1$ was washed with 40 ml. '0·45 Am_2SO_4, *p*H 7'. To the mother liquor a mixture of 3·4 ml. 0·1 N-H_2SO_4 and 2·4 ml. 'Am_2SO_4, *p*H 5' was added, but the solution remained transparent (*p*H=5·03) even after an addition of 1·6 ml. 'Am_2SO_4, *p*H 5'. By adding a further 3 ml. 'Am_2SO_4, *p*H 5' the precipitate $g_4^5\gamma_1$ was obtained. It was washed twice with 25 ml. '0·50 Am_2SO_4, *p*H 5'. The next precipitate was not obtained until a salt concentration corresponding to '0·60 Am_2SO_4' was reached. $ga_2^7\gamma_1$ was washed with 30 ml. '0·65 Am_2SO_4, *p*H 7'. On acidifying the mother liquor, no precipitate occurred until the *p*H was about 4·7. $a_1\gamma_1$ was washed with 50 ml. '0·65 Am_2SO_4, *p*H 5'. By further acidification to *p*H 4·1, $a_2\gamma_1$ was obtained. This was washed with 50 ml. '0·65 Am_2SO_4, *p*H 5'. Addition of solid ammonium sulphate did not produce any further precipitate.

From Table 38, p. 119, it is seen that the umbilical serum contains considerable amounts of euglobulin (Howe) but very little fetuin, just the opposite to the case of fœtal serum from cow and sheep.

C. Serum from Other Species.

a. Adult and Fœtal Serum from Rabbit.

For the sake of comparison, the maternal serum and the corresponding fœtal serum from rabbit were fractionated.

Fractionation Experiment No. 28. 50 ml. of maternal rabbit serum was first diluted with the same volume of 0·2 M-NaCl. Precipitates were then successively taken at the following concentrations of saturated ammonium sulphate: '0·33', '0·37', '0·40', '0·45', '0·50', '0·55', and '0·60'. These gave the corresponding fractions $g_1\mu_1$ to $ga_2\mu_1$. Each precipitate was washed with 50 ml. of its corresponding wash-solution. The wash-solutions were '0·37', '0·42', '0·45', '0·50', '0·55', '0·60' and '0·65 Am_2SO_4' respectively. The mother liquor from $ga_2\mu_1$ was acidified to *p*H 4·7 by the addition of 1·6 ml. 1 N-H_2SO_4. The precipitate $a_1\mu_1$ was washed with 50 ml. '0·65 Am_2SO_4'. The addition of solid ammonium sulphate only produced a slight turbidity.

[1] The author is greatly indebted to Professor J. Naeslund of the Obstetric Clinic, University Hospital, Upsala, for providing this blood.

TABLE 41. COMPOSITION OF DIFFERENT FRACTIONS FROM FOAL SERUM.

Fraction	Milligrams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram				Inhomogen. material not in diagram		Grams of protein per 100 ml. of serum
			s_{20}	Δn	%	mg.	s_{20}	Δn	%	mg.	s_{20}	Δn	%	mg.	s_{20}	Δn	%	mg.	Δn	%	g.	%	mg.		
$g_2\ \lambda_1$	18	146	2·47	75	51	9·2					6·36	15	10	1·8	19·0	8	6	1·1				33	5·9		0·14
$g_3\ \lambda_1$	7	77	3·21	28	36	2·5					7·11	9	12	0·8		2	3	0·2	32	41	2·9	8	0·6		0·06
$g_4\ \lambda_1$	5	54					4·38	36	67	3·4						1	2	0·1				31	1·5		0·04
$g_5\ \lambda_1$	10	100					5·03	67	67	6·7					20·9	8	8	0·8	5	5	0·5	20	2·0		0·08
$ga_1\ \lambda_1$	16	146	3·11	20	14	2·2	4·64	50	34	5·5						10	7	1·1				45	7·2		0·13
$a_1\ \lambda_1$	356	508					4·27	144	28	99·7									31	6	21·3	66	235·0		2·85
'Total globulin'	40				29	11·7			25	10·1			7	2·6			5	2·2		9	3·4	25	10·0		0·32
Total	412				3	13·9			28	115·3			1	2·6			1	3·3		6	24·7	61	252·2		3·30

TABLE 42. COMPOSITION OF DIFFERENT FRACTIONS FROM FŒTAL SHEEP SERUM.

$g_2^7\ \iota_1$	18	162	3·15	80	50	9·0					5·75	7	4	0·7								46	8·3		0·36
$g_3^7\ \iota_1$	20	178	3·06	118	66	13·2																34	6·8		0·40
$g_4^5\ \iota_1$	19	186	3·19	50	27	5·1									20·6	12	6	1·2	56	30	5·7	37	7·0		0·38
$a_3\ \iota_1$	70	350					4·31	248	71	50·0												29	20·0		1·40
'Total globulin'	57				48	27·3							1	0·7			2	1·2		10	5·7	39	22·1		1·14
Total	127				21	27·3			39	50·0			1	0·7			1	1·2		5	5·7	33	42·1		2·54

From Table 39 it is seen that even the serum from a pregnant rabbit contains definite, although not large, amounts of fetuin. Another remarkable point is the presence, in the fractions corresponding to total globulin, of as much as 8 per cent of a substance sedimenting with the same velocity as albumin (*X*-protein?).

Fractionation Experiment No. 29. The serum used in this experiment was obtained from the pooled blood of eleven fœtus taken from the same pregnant rabbit as above just before the expected parturition. The total volume of the fœtal serum was 6 ml. It showed a strong red colour owing to haemolysis.

5 ml. of fœtal serum was diluted with the same volume of 0·2 M-NaCl. The first precipitate, $g_2\nu_1$, was obtained after the addition of 5·9 ml. '1·0 Am_2SO_4'. It was washed with 10 ml. '0·42 Am_2SO_4'. The next precipitate, $g_4\nu_1$, did not occur until a further 2·3 ml. of the ammonium sulphate solution had been added. It was washed with 10 ml. '0·50 Am_2SO_4'. The third precipitate, $ga_1\nu_1$, appeared after the addition of a further 3 ml. of ammonium sulphate solution. It was washed with 10 ml. '0·60 Am_2SO_4'. The albumin was obtained after full saturation of the mother liquor, it was strongly coloured by haemoglobin.

As will be seen from Table 40, rabbit fœtal serum contains rather little fetuin, but comparatively much normal globulin. The amount of inhomogeneous material is quite considerable.

b. Foal Serum.

In connection with the study of fetuin, it was of interest to investigate the presence of this protein in the serum from different newborn or very young animals. The fractionation of a foal serum will now be described.

Fractionation Experiment No. 30. The serum used in this experiment was from a one day old foal. It had been stored for several years.[1] When it had thawed, a small sediment, which appeared at the bottom of the bottle, was discarded. The remainder of the serum, 12·5 ml., was first diluted with 12·5 ml. 0·2 M-NaCl and then mixed with 12·5 ml. '1·0 Am_2SO_4'. As no precipitate appeared, a further 2·25 ml. of the salt solution was added. The resulting precipitate, $g_2\lambda_1$, was washed with 25 ml. '0·42 Am_2SO_4'. By adding, successively, 2, 3·75, 4·5 and 5·5 ml. 'Am_2SO_4' to the mother liquor, the following precipitates were obtained: $g_3\lambda_1$, $g_4\lambda_1$, $g_5\lambda_1$ and $ga_1\lambda_1$. Each precipitate was washed with 25 ml. of its corresponding wash-solution. The wash-solutions were '0·45', '0·50', '0·55' and '0·60 Am_2SO_4', respectively. By adding 7 ml. 'Am_2SO_4' and 2·5 ml. 0·1 N-H_2SO_4 the *p*H was changed to 4·7 and $a\lambda_1$ was precipitated. The addition of solid ammonium sulphate produced only a slight turbidity.

[1] The serum was placed at the disposal of the author by Dr. G. S. Adair. It had been kept at −15° C in Cambridge for about one year. It was then transferred to Upsala in a thermosbottle with ice and finally stored at −8° C for five years. It appeared to be completely fresh.

In the sedimentation diagrams from the fractions belonging to 'total globulin', the predominant component is the fetuin. The normal globulin is present in definite although not very large quantities. The most slowly sedimenting peak in $g_4\lambda_1$ and $g_5\lambda_1$ is very broad and undoubtedly of a composite nature. It seems likely that fetuin is responsible for a large part of this peak.

c. Fœtal Serum from Sheep.

Fractionation Experiment No. 31. The serum used in this experiment originated from a 133 days old sheep fœtus.[1] 5 ml. of serum was diluted with the same amount of 0·2 M-NaCl* and of '1·0 Am_2SO_4, *p*H 7', but no precipitate occurred. After the addition of a further 0·9 ml. of the latter solution, $g_2^1\iota_1$ came down after a short time. This was allowed to stand for half an hour (*p*H of solution 6·96). The precipitate was washed twice with 5 ml. '0·42 Am_2SO_4, *p*H 7'. The addition of 0·8 ml. 'Am_2SO_4, *p*H 7' produced $g_3^1\iota_1$, which was washed twice with 5 ml. '0·45 Am_2SO_4, *p*H 7'. The mother liquor was acidified with a mixture of 1·18 ml. 0·1 N-H_2SO_4 and 0·8 ml. 'Am_2SO_4, *p*H 5' but no deposit was formed until after a further addition of 1·5 ml. of the ammonium sulphate solution. The precipitate $g_4^5\iota_1$ was washed twice with '0·50 Am_2SO_4, *p*H 5'. The addition of ammonium sulphate was carried out according to the usual scheme, but not until a salt concentration of '0·60 Am_2SO_4' had been reached and the *p*H had been changed to 4·7 did the solution become slightly turbid. After the addition of solid ammonium sulphate the precipitate $a_3\iota_1$ was finally obtained.

As shown in Table 42, the amounts of normal globulin and of the 20-component in this fœtal serum were almost negligible. The fetuin predominates in the sedimentation diagram, but the quantity of inhomogeneous material is much higher than usual. This is probably due to the long storage of the serum.

D. Fractionation of Delipidated Serum.

For the study of some of the properties of the serum proteins it is necessary to remove the lipids. As has been described earlier on p. 46 the extraction of these substances produce great changes in the sedimentation diagrams of serum, and it may therefore be of some interest to study the fractionation of delipidated sera. McFarlane (1935, e) examined the 'total globulin' and the albumin prepared from human serum extracted by the Hardy-Gardiner technique and found that both fractions contained large quantities of inhomogeneous material. On the other hand, Hewitt (1927) has found that 'reconstituted plasma

[1] See note p. 127.

can still be caused to clot and albumins may be crystallized'. This finding has been confirmed by Sørensen (1930), who considers the extraction of the lipids from serum by this method to involve only a purification of the proteins but no real chemical splitting. The ultracentrifugal experiments do not verify this conclusion. Some of the serum proteins, at least, are undoubtedly seriously changed by such treatment, while others, for instance a part of the serum albumin and the fetuin, seem to be comparatively little affected.

a. Calf Serum.

Fractionation Experiment No. 32. The same serum as used in Fractionation Experiment No. 14 was extracted with acetone and ether. All the processes, mixing, centrifuging, *etc.*, were carried out in the open air on a cold winter day (temperature: $-10°$ to $-15°$ C). 16 ml. of serum, cooled to $+2°$ C, was injected into each of 6 centrifuge tubes containing 90 ml. acetone ($-14°$ C) by means of a syringe through a capillary. Immediately after the mixing, while the syringe was refilled, the tube was vigorously shaken (gloves!). As soon as the last tube had been mixed and shaken (about 5 min.), the precipitate was centrifuged down quickly, the supernatant poured off and the precipitate resuspended in 6×90 ml. dry ether. It was allowed to stand for half an hour with occasional shaking and finally centrifuged down. The precipitate was then resuspended in acetone (at 0° C), occasionally shaken and after half an hour centrifuged down. For the remaining part of the extraction two different courses were followed. Four of the precipitates, ε_{11A}, were twice treated each with 90 ml. dry ether, whereas two of them, ε_{11B}, were each first extracted with a mixture of 30 ml. dry ether and 60 ml. acetone and then twice treated with 90 ml. dry ether. The tubes with the precipitates were then placed in a vacuum desiccator, which was taken outdoors in order that it might receive the tubes directly in the cold. The tubes were then allowed to attain room temperature. Finally, the precipitates, ε_{11A} and ε_{11B}, were both extracted with dry ether in a Soxhlet apparatus. The dry protein powder dissolved easily and almost completely in water.

Of ε_{11A} 3·7 g. was dissolved in 50 ml. water and dialysed against 0·2 M-NaCl, which resulted in 55 ml. of solution with $\Delta n = 990$. 50 ml. of the solution was diluted with 0·2 M-NaCl to 200 ml. Addition of 100 ml. 'Am_2SO_4, *p*H 7' gave no precipitate; after a further 18 ml. had been added $g_2^7\,\varepsilon_{11A}$ came down. Acidification to *p*H 5 gave $g_2^5\,\varepsilon_{11A}$. Attempts were made to get precipitates at the following concentrations, but only a few were obtained: at '0·40 Am_2SO_4, *p*H 7', no precipitate; at '0·40 Am_2SO_4, *p*H 5'. $g_3^5\,\varepsilon_{11A}$; at '0·45 and 0·50 Am_2SO_4, *p*H 7 and 5' and at '0·55 Am_2SO_4, *p*H 7' no precipitates; at '0·55 Am_2SO_4, *p*H 5', $ga_1^5\,\varepsilon_{11A}$; at '0·60 Am_2SO_4' no precipitates were obtained at *p*H 7, 5·1 and 4·7, but at *p*H 4·1 $a_2\,\varepsilon_{11A}$ was obtained. Saturation with ammonium sulphate gave only a slight turbidity. The larger precipitates were washed twice with 100 ml. of a solution having the proper *p*H and more concentrated by '0·05 Am_2SO_4' than the supernatant; for the smaller precipitates 50 ml. was used instead of 100 ml. The results from the ultracentrifugal examination of the different fractions of ε_{11A}, ε_{11B} and the reconstituted serum ε_{11A} are summarized in Table 43.

TABLE 43. COMPOSITION OF DIFFERENT FRACTIONS OBTAINED FROM DELIPIDATED CALF SERUM.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material					Grams of protein per 100 ml. of serum
																			in sedimentation diagram			not in diagram		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
ε_{11A}	2·46																							
$g_2^7\ \varepsilon_{11A}$	0·58	644	3·13	87	14	0·081					6·63	72	11	0·064								75	0·435	1·00
$g_2^5\ \varepsilon_{11A}$	0·62	472	3·07	95	20	0·124					6·31	26	5	0·031					103	22	0·136	53	0·329	1·07
$g_3^5\ \varepsilon_{11A}$	0·47	556	3·25	62	11	0·052	4·20	128	23	0·108	6·12	66	12	0·056					96	17	0·080	37	0·174	0·81
$ga_1^5\ \varepsilon_{11A}$	0·20	405					4·34	235	58	0·116	6·29	51	13	0·026								29	0·058	0·35
$a_2\ \varepsilon_{11A}$	0·14	256					4·30	213	83	0·116												17	0·024	0·24
Total	2·01				13	0·257			17	0·340			9	0·177						10	0·216	51	1·020	3·47
$g_2^7\ \varepsilon_{11B}$	0·14	381	2·99	29	8	0·011	4·09	8	2	0·003	6·89	83	22	0·030					55	14	0·020	54	0·076	0·42
$g_2^5\ \varepsilon_{11B}$	0·08	295	3·09	55	19	0·015					6·42	31	10	0·008					8	3	0·002	68	0·055	0·24
$g_3^5\ \varepsilon_{11B}$	0·14	445	2·99	108	24	0·034					6·45	30	7	0·010					79	18	0·025	51	0·071	0·43
$g_4^7\ \varepsilon_{11B}$	0·05	194	3·19	65	34	0·017					6·65	78	40	0·020								26	0·013	0·15
$g_4^5\ \varepsilon_{11B}$	0·18	326	3·47	40	12	0·022	4·31	55	17	0·031	6·38	44	14	0·025					76	23	0·041	34	0·061	0·54
$g_5^5\ \varepsilon_{11B}$	0·18	334					4·24	203	61	0·110	6·19	32	9	0·016								30	0·054	0·55
$a_2\ \varepsilon_{11B}$	0·11	287					4·36	181	63	0·069												37	0·041	0·33
Total	0·88				11	0·099			24	0·213			13	0·109						10	0·088	42	0·371	2·66

Of ε_{11B} 1·5 g. was dissolved in 25 ml. water and dialysed against 0·2 M-NaCl, giving 26·5 ml. solution with $\Delta n = 950$. 25 ml. of the solution was diluted with 0·2 M-NaCl to 100 ml. Precipitations were attempted at the same ammonium sulphate concentrations and *p*H values as with ε_{11A}. It is seen from Table 43 that a few more precipitates were obtained than in the latter case. In Table 44 the results for the relative amount of the individual components (in per cent) may be compared for the four different fractionation experiments on the same serum.

TABLE 44. RELATIVE AMOUNTS OF VARIOUS COMPONENTS FROM DIFFERENT FRACTIONATION EXPERIMENTS WITH THE SAME CALF SERUM.

ε_{12} and ε_{13} are from unextracted serum (Tables 19—20), while ε_{11A} and ε_{11B} have been delipidated before the fractionation (Table 43).

Serum fractions from	Fetuin	'Albumin'	Globulin	20-comp.	Inhomogeneous material	
					in sed. diagram	not in sed. diag.
ε_{12}	10	(45)[1]	6	2	1	(36)[1]
ε_{13}	12	(46)[1]	7	2	0	(33)[1]
ε_{11A}	12	17	9	0	11	51
ε_{11B}	11	24	13 (10)[2]	0	10	42 (45)[2]

It is seen from Table 44 that the fetuin appears to be completely unaffected by the extraction of the lipids, while the 20-component is destroyed. Blix (1941) also found that the α-globulin peak disappeared from the electrophoresis diagrams for lipid-free human sera at *p*H 6·1 and 7·4, but when the electrophoresis experiment was carried out at *p*H 8, the presence of this peak could often just be detected. The 'albumin' part of the serum has been considerably reduced, while the 'globulin' has been somewhat increased. This conforms with the result given in Table 2, p. 48, where electrophoretically isolated human serum albumin, after the removal of the lipids, showed an 'albumin' as well as a 'globulin' peak in the sedimentation diagrams. This is a strong indication that the '*X*-protein' complex (β globulin) is often hidden in the 'albumin' peak. When the lipids are extracted, the *X*-protein splits

[1] Some of the albumin fractions were not examined in the ultracentrifuge and the proportion between 'albumin' and 'inhomogeneous material' could consequently only be estimated.

[2] Corrected values, after $g_{\frac{1}{2}}^{7}\,\varepsilon_{11B}$ and $g_{\frac{1}{2}}^{5}\,\varepsilon_{11B}$ have been spun in the air-driven centrifuge.

TABLE 45. THE EFFECT OF SPINNING THE DIFFERENT FRACTIONS FROM DELIPIDATED SERUM IN THE AIR-DRIVEN CENTRIFUGE FOR 6 HOURS AT 27,000 R.P.M.

Fraction	Δn		Inhomogen. heavy material in per cent	Δn from sedimentation diagram		Consistency of bottom fraction in air-driven centrifuge *etc.*
	Initial	After centrifuging		Before removal of heavy material	After removal of heavy material	
			[1]			
$g_2^7\ \varepsilon_{11A}$	644	248	61 (75)	159	166	
$g_2^5\ \varepsilon_{11A}$	708	349	51 (53)	224	216	
$g_3^5\ \varepsilon_{11A}$	556	349	37 (37)	356		
$ga_1\ \varepsilon_{11A}$	405	365	10 (29)	286		
$g_2^7\ \varepsilon_{11B}$	381	85	78 (54)	175	80	massive pellet
$g_2^5\ \varepsilon_{11B}$	295	77	74 (68)	94	68	"
$g_3^5\ \varepsilon_{11B}$	445	201	55 (51)	217		"
$g_4^7\ \varepsilon_{11B}$	194	100	48 (26)	143		small pellet +yellow syrup
$g_4^5\ \varepsilon_{11B}$	326	186	43 (34)	215		"
$g_5^5\ \varepsilon_{11B}$	334	264	21 (30)	235		syrupy
$a_1\ \varepsilon_{11B}$	287	178	38 (37)	181		yellow pellet

into 'albumin', globulin and inhomogeneous material. In the sedimentation diagram this gives rise to a decreased 'albumin' peak and an increased globulin peak.

The large quantities of inhomogeneous material in ε_{11A} and ε_{11B} made it reasonable to investigate these fractions a little further. They were therefore spun in the air-driven quantity centrifuge at 27,000 R.P.M. for 6 hours (see Table 45). In most cases a massive transparent or translucent pellet was formed at the bottom of the centrifuge tube. The occurrence of such elastic jellies must be due to a profound change in the corresponding part of the proteins. The sediment from a 'globular' protein has always a more or less syrupy consistency, whereas these pellets greatly resemble those obtained after spinning

[1] Values in bracket refer to 'Inhomogeneous material not accounted for in sedimentation diagram' before the spinning of the solution at 27,000 R.P.M., while the first figures are calculated from the Δn's in column 2 and 3.

down the gelatine from a jelly in the ultracentrifuge. The proteins constituting the pellet have undoubtedly changed their state from 'globular' to 'fibrous' and such an alteration is usually classified under the general heading of denaturation.

It is seen from the table that in some cases the Δn, calculated from the sedimentation diagram, has the same value after the removal of the heavy material in the air-driven centrifuge as before. In other instances, Δn was much lower after the spinning of the solution in the air-driven centrifuge. Inspection of the sedimentation diagrams for the latter solutions also showed very asymmetrical globulin peaks, indicating the presence of more inhomogeneous material in these peaks than was at first supposed.

b. Human Serum.

The ultracentrifuge runs on human sera, deprived of most of their lipids according to the method of Horsfall and Goodner (1935), showed that great changes had taken place with the serum proteins. In order to investigate whether another method would be more gentle, the extraction was made on lyophilized serum. Arrhenius (1944), working with cow serum, has found that such sera may yield clear solutions with protein concentrations up to 10 per cent, but their dielectric behaviour is changed. He also supposes that the internal structure of the serum proteins is altered.

Fractionation Experiment No. 33. Human serum was frozen at $-15°$ C and dried in vacuo over $CaSO_4$ in the chryochem apparatus briefly described by Widström (1944). The dry, fine powder was extracted twice in the cold room (about $+4°$ C) for two hours, first with absolute alcohol (100 times its own weight) and then twice with dry ether. It was finally extracted in the Soxhlet apparatus in the usual way. The ether was removed from the extracted powder in a desiccator. The dried serum dissolved slowly in water and was dialysed against 0·2 M-NaCl. The solution was rather turbid and two different procedures were tried for removing the turbidity. In the first, 50 ml. of the reconstituted serum was spun in the air-driven centrifuge for 2 hours at 27,000 r.p.m. A middle fraction (41 ml.) with $\Delta n = 530$ was reserved and used for the fractionation experiment after dilution to 50 ml. This was called α_{7A}. In the second experiment, α_{7B}, 110 ml. of the cold reconstituted serum was extracted twice in the cold room ($+4°$ C) with 110 ml. ice-cold ether. The top and bottom part from the separation in a Swedish angular centrifuge were discarded; the middle fraction, 83 ml. with $\Delta n = 643$, was diluted to 100 ml. with 0·2 M-NaCl and fractionated with ammonium sulphate.

Attempts were made to obtain precipitates at the following ammonium sulphate concentrations: at '0·33, *p*H 5', $g_1^5\,\alpha_{7B}$; at '0·33, *p*H 7', no precipitate; at '0·37, *p*H 5'

TABLE 46. COMPOSITION OF DIFFERENT FRACTIONS OBTAINED FROM DELIPIDATED HUMAN SERUM.

Fraction	Grams of protein	Δn in run	Fetuin				'Albumin'				Globulin				20-component				Inhomogen. material in sedimentation diagram					Grams of protein per 100 ml. of serum
																			in			not in		
			s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	s_{20}	Δn	%	g.	Δn	%	g.	%	g.	
$g_1^5\ \alpha_{7A}$	0·10	452	2·29	2			4·18	27	6	0·006	6·86	132	29	0·029					43	10	0·010	55	0·055	
$g_2^5\ \alpha_{7A}$	0·15	595	3·26	14	2	0·003	4·50	68	12	0·018	6·34	216	36	0·054	14·1	22	4	0·006	41	7	0·011	39	0·058	
$g_2^7\ \alpha_{7A}$	0·02	146					4·67	18	12	0·002	7·45	50	34	0·007	20·6	3	2	0·001				52	0·010	
$g_3^7\ \alpha_{7A}$	0·47	627					3·95	405	65	0·305						10	1	0·005	51	8	0·038	26	0·122	
$g_3^5\ \alpha_{7A}$	0·02	248					4·59	129	52	0·010	6·78	49	20	0·004								28	0·006	
$g_1^5\ \alpha_{7B}$	0·14	547					4·43	26	5	0·007	6·94	82	15	0·021								80	0·112	
$g_2^5\ \alpha_{7B}$	0·17	579	3·14	16	3	0·005	4·58	46	8	0·014	6·35	277	48	0·082	15·8	11	2	0·003	33	5	0·008	34	0·058	
$g_2^7\ \alpha_{7B}$	0·07	365					4·23	50	14	0·010	6·48	207	57	0·040	17·5	7	2	0·001	44	12	0·008	15	0·011	
$g_3^7\ \alpha_{7B}$	0·14	396		6	1	0·001	4·51	60	15	0·021	6·81	130	33	0·046	18·8	11	3	0·004	24	6	0·009	42	0·059	
$g_3^5\ \alpha_{7B}$	0·04	248					4·59	129	52	0·021	6·78	49	20	0·008								28	0·011	
$g_5^7\ \alpha_{7B}$	0·05	209					4·55	104	50	0·025									68	32	0·016	18	0·009	
$g_5^5\ \alpha_{7B}$	0·75																							
$ga_2^5\ \alpha_{7B}$	0·06																							

$g_2^5\,\alpha_{7B}$; at '0·37, *p*H 7', $g_2^7\,\alpha_{7B}$; at '0·40, *p*H 7', $g_3^7\,\alpha_{7B}$; at '0·40, *p*H 5'. $g_3^5\,\alpha_{7B}$; at '0·45, *p*H 7 and 5', no precipitates; at '0·50, *p*H 7', $g_5^7\,\alpha_{7B}$; at '0·50, *p*H 5', $g_5^5\,\alpha_{7B}$; at '0·55, *p*H 7 and 5' and '0·60, *p*H 7', no precipitates. Finally the precipitate $ga_2^5\,\alpha_{7B}$ was obtained at '0·60, *p*H 5'.

With the other solution, α_{7A}, attempts were made to get precipitates at the same concentrations as above up to and including '0·45 Am_2SO_4, *p*H 7'. The fractions obtained are given in Table 46 together with the results of the ultracentrifugal examination of most of these.

It will be seen from the table that the relative amount of inhomogeneous material in α_7 is definitely less than in the preceding experiment, ε_{11}. A 20-component is also present in both α_{7A} and α_{7B}, but there is about three times as much in the former as in the latter, where the reconstituted serum was extracted with ether in the cold. The 20-component appears from the sedimentation diagrams to be less homogeneous after the extraction than before. Its presence may be an indication either that the method is mild or that the 20-component is more stable in human than in calf serum (*cf.* Blix 1941). The large quantities of albumin precipitated in α_{7A} at '0·40 Am_2SO_4, *p*H 7' are rather astonishing; in α_{7B} the main part comes down at '0·50 Am_2SO_4, *p*H 5', which seems more reasonable. The appearance of the albumin in the first globulin fractions may be due to the fact that these precipitates were not washed, but only centrifuged sharply down.

Taken as a whole, the amounts of albumin in the extracted sera correspond to the normal value, but the globulin is lower and the inhomogeneous material correspondingly higher.

4. DISCUSSION AND CONCLUSIONS.

The fractionation experiments described in this chapter have all confirmed the old experience that it is not an easy matter to prepare pure serum proteins by fractionation with ammonium sulphate. They have also shown that the ultracentrifuge is an instrument well fitted for following the progress in this purification, especially that of the serum globulins. It has also been used in the U. S. A. for controlling the large scale production of albumin and immune globulin from human serum (*cf.* Williams *et al.*, 1944).

The attempts to obtain at least ultracentrifugally pure globulin fractions by repeated precipitation with ammonium sulphate were only successful with regard to the least soluble globulin (the euglobulin in Howe's nomenclature). All such fractions investigated have been found

to contain certain quantities of inhomogeneous heavy material, which could, however, be removed by sedimentation for some hours in the air-driven centrifuge at 27,000 R.P.M., while the remainder appeared homogeneous in the ultracentrifuge. By dialysis against dilute salt solutions or against water it is possible, however, to divide the globulin solution into an euglobulin precipitate and a pseudoglobulin solution. A smaller or greater part of the euglobulin may be dissolved in 0·2 M-NaCl and this solution as well as the corresponding pseudoglobulin solution appear to consist of normal pure globulin in the sedimentation diagrams. In addition to the component visible in the sedimentation diagram they usually contain small amounts of inhomogeneous heavy material, the euglobulin generally showing more than the pseudoglobulin. Part of the euglobulin formed by dialysis is often not soluble in 0·2 M-NaCl, but may be dissolved in more concentrated salt solutions (sometimes at 40° C). The sedimentation diagrams from such solutions generally show that they consist of a wide variety of inhomogeneous material and their proteins are best classified as irreversibly degraded or denatured globulins. It may also be mentioned that according to Grönwall (1940, p. 97) 'A fractionation of serum globulin into pseudoglobulin and euglobulin, by dialysis or dilution (with water), is a irreversible process'.

If the euglobulin, soluble in 0·2 M-NaCl, is dialysed against dilute salt solutions or water, it may again be divided into an euglobulin precipitate and a pseudoglobulin solution. In a similar way, if the pseudoglobulin solution is re-precipitated with ammonium sulphate, dissolved in 0·2 M-NaCl and dialysed against dilute salt solutions or water, it may also be separated into an euglobulin precipitate and a pseudoglobulin solution. Such a behaviour of the ultracentrifugally pure serum globulin may for instance be explained in the following manner: The protein solution contains both intact and degraded serum globulins, the latter partly formed during the purification process. When the solution is dialysed against dilute salt solutions or water, a large part of the degraded proteins precipitate at an early stage, and when the salt concentration eventually falls below the value corresponding to the solubility of the other globulins in the bag, the precipitation of these proteins commences. If this explanation is correct, the main difference between the euglobulins and the pseudoglobulins (prepared from ultracentrifugally pure globulin) should in this case be the presence of larger quantities of degraded globulins in the

former than in the latter. The large boundary spreading of $P_{II}\,\delta_1$ indicates, however, that the pseudoglobulin is electrochemically inhomogeneous and, to judge from Cohn (1941) there seem also to be some electrochemical differences between the various fractions: 'When the γ-globulin fraction is further fractionated by isoelectric precipitation into various euglobulins and pseudoglobulins, their electrophoretic mobilities prove not to be identical, although all are generally within the area covered by the probability curve of the mean mobility of the γ-globulin'.

In this connection it is also of interest to note that Grönwall (1945) has recently found that the antibody protein in antipneumococcus rabbit serum is present in *all* the globulin precipitates obtained with sodium sulphate. A further fractionation of the three globulin fractions obtained was made by dialysis against water ($6 < p\mathrm{H} < 6{\cdot}5$). The euglobulin precipitates were only partly soluble in 0·8 per cent NaCl and the insoluble parts were discarded. Determination of the percentage of antibody nitrogen in the six solutions showed that it was about 40 in the three euglobulins, while it increased from 37 in the least soluble to 62 in the most soluble pseudoglobulin. From the analysis of the soluble fractions it appears that the insoluble euglobulins could not have contained any antibody.

The globulin fractions obtained between '0·37' and '0·50 Am_2SO_4' yielded in no case pure globulin components and several re-fractionations with ammonium sulphate produced only small changes in the relative proportions of the various components. From the least soluble fractions it was possible after several re-precipitations to gain some comparatively pure 'normal globulin'. A small amount of fetuin (or '*X*-protein'?) was, however, almost impossible to remove. From the most soluble fractions a small amount of ultracentrifugally almost pure 'albumin' could be prepared after several re-fractionations.

All the re-fractionation experiments showed one common feature in so far as the first fraction generally contained a very large part of the total globulin precipitated (*cf.* Tables 8, 20 and 22). Those fractions only which might be suspected to consist partly of '*X*-protein' showed a different behaviour (*cf.* Tables 10 and 30). This may be explained by the solubility increasing effect of the serum albumin in serum (*cf.* Grönwall, 1940, p. 90 ff.) or of the '*X*-protein'.

The fractions from cow serum just mentioned corresponded to g_2, and mainly g_3, in Fractionation Experiment No. 1 (p. 63). It is seen

from Fig. 24 (p. 64) that these fractions, besides the γ-globulin, are very rich in both α- and β-globulin. As, however, the serum was not diluted in this experiment before the addition of the concentrated ammonium sulphate (final serum concentration: 0·6 $c_0 \rightarrow 0{\cdot}5\ c_0$) it may be expected that more of the albumin and the γ-globulin was present in the '*X*-protein' complex (α- or β-globulin) than in the later experiments where the serum was first diluted with an equal volume of saline (final serum concentration 0·32 $c_0 \rightarrow 0{\cdot}25\ c_0$). Svensson (1941) found considerable quantities of β-globulin in the '0·40 Am_2SO_4' fractions from horse and rabbit, and somewhat less in pig serum. The fraction between '0·40' and '0·55 Am_2SO_4' gave 79 per cent α-globulin from pig and 49 per cent from horse, while for β-globulin the figures were 14 and 36 per cent respectively (serum concentration 0·6 $c_0 \rightarrow \sim 0{\cdot}5\ c_0$). From Table 3 (p. 56) it is seen that Cohn *et al.* (1940, a) also found α- and β-globulins together with γ-globulin in the corresponding fractions from horse serum, but the relative amounts of the different components are not given (serum concentration in experiment 0·5 c_0). In a recent paper Cohn *et al.* (1944) have given the distribution of the proteins of human plasma in 6 different fractions (no details are given about the serum dilution or precipitation range, but it appears that their fractions III and IV were within the range '0·37' to '0·50 Am_2SO_4'. Furthermore the serum was probably not much diluted). From their values the following percentages are calculated:

	Alb.	α	β	γ	Fibr.
Fraction II + III . . .	4	11	38	37	10
Fraction IV	10	56	32	2	0

In Fractionation Experiments Nos. 21 and 22 (pp. 116 and 117) with human sera the relative concentrations of both the α- and the β-globulin were high (serum concentration $\sim 0{\cdot}3\ c_0$).

The difficulty of achieving a proper separation of the different components in the range '0·37' to '0·50 ('0·60') Am_2SO_4', may be explained by the presence of α- and β-globulin in these fractions. According to the results obtained in Chapter I we must assume that at least a part of the β- (or α-) globulin consists of the compound formed from albumin, globulin, lipids, *etc.* (the '*X*-protein'). Depending on the concentrations of the various components in the solution this '*X*-protein' will dissociate or take up some of the other components. It must therefore be expected that the results will be different when the frac-

tionations are carried out with a rather concentrated serum and with a diluted serum. In the former case more of the '*X*-protein' and less of the γ-globulin are precipitated and in the latter case *vice versa*. This is also in accordance with the frequent finding in serum analysis that the ratio globulin/albumin apparently decreases with increasing dilution of the serum.

It may thus be concluded that when γ-globulin or albumin is to be prepared, it is advantageous to make the precipitations from fairly diluted sera, while such compounds as the '*X*-protein' are best obtained from concentrated sera. Quite the opposite procedure has been used by Jameson and Alvarez-Tostado (1943) in their preparation of horse γ-globulin. They worked with concentrated serum and precipitated the 'γ-globulin' with potassium citrate. They also find that in addition to the main γ-component 'there is an appreciable amount of beta and alpha in all the fractions'!

As the contact with strong salt solutions always produces some inhomogeneous heavy material, it is important that each step in the fractionation process brings about a definite improvement in the purity of one of the components. For this reason the usual procedure of re-precipitation with ammonium sulphate was dropped for the fractions between '0·37' and '0·50 Am_2SO_4'.

The fractionation with ammonium sulphate was tried at different *p*H values between 5 and 7. The risk of including albumin in the globulin fractions was found to be smaller at the latter *p*H. It was observed that part of the serum pigments followed the globulin fractions when, at room temperature (18° C), the *p*H of the precipitating solution was kept at 5·5 or below. Since the pigments in electrophoresis seem mainly to follow the β- and α-globulins, it is reasonable to suppose that the colour of the globulin fractions obtained below *p*H 5·5 may be due to the presence of the '*X*-protein'. In the other cases the greater part of the pigments came down with the *ga* or the first *a* fractions which contain great quantities of α- and β-globulin. If the temperature after the acidification to *p*H 5 was kept below + 5° C, the globulin solutions would generally be very little coloured. The coloured '*X*-protein' precipitated below *p*H 5·5 at room temperature is, however, probably denatured and changed to inhomogeneous material, since the coloured globulin solution could usually be made almost water-clear by spinning in the air-driven centrifuge at 27,000 R.P.M. for some hours. After such a run the inhomogeneous material

had collected as a strongly coloured pellet at the bottom of the centrifuge tube. The presence of such inhomogeneous material may quite well be responsible for the often experienced difficulty in obtaining results from electrophoresis experiments on serum fractions in accordance with those from ultracentrifugal runs.

Fractionation by dialysis against dilute salt solutions and water was also tried, and it was found that by suitable dialysis almost all the 20-component could be removed together with part of the globulin. At the same time, however, the relative amount of the fetuin increased, and at the end of the dialysis it was almost the same in the precipitate as in the supernatant. Similarly dissolution of the precipitates and new dialysis could improve only to a limited extent the purity of the globulin and the 20-component. The method may be of value when the problem is to cut down the amount of the 20-component or to increase the relative amount of fetuin.

Dialysis of the whole serum against dilute salt solutions before precipitation with ammonium sulphate may in some cases produce a comparatively pure globulin precipitate, provided that the *p*H of the solution is kept above 6. If the solution is acidified to *p*H 5 or below, considerable quantities of inhomogeneous material (denatured '*X*-protein'?) are formed. Sometimes this inhomogeneous material is precipitated during dialysis while on other occasions the main part is deposited together with the first normal globulin precipitate. This method seems only to be of value when fetuin is to be prepared from calf serum.

It was attempted to fractionate the serum globulin in a way other than the usual. The *g* and the *ga* fractions were first precipitated and the adhering albumin washed away as thoroughly as possible. The precipitate was then extracted with solutions having successively decreasing ammonium sulphate concentration. At each step the extraction was repeated until the last extract contained only insignificant amounts of protein. It was hoped that the distribution of the components would be such that a reprecipitation with ammonium sulphate could yield comparatively pure fractions. The long contact with ammonium sulphate during the extraction is, however, a serious drawback, and, as no definite advantages were gained, the method cannot be recommended.

In the search for better ways of preparing pure serum proteins, it was thought worth while to study the fractionation of calf serum, as

it was to be expected that the globulin composition was somewhat different from that of cow serum. This study led to the isolation of the new globulin fetuin (Pedersen, 1944). The presence of this protein in other sera will be discussed later in this section. It seems that the method may also be advantageous in the preparation of other serum proteins.

Finally, the precipitation at alternating *p*H values was tested. So far this method offers the best possibilities in the fractionation of the serum proteins, and it seems likely that it may be still further improved by suitable variation of the precipitation temperature. The only objection the author can see to this method, as used in the present investigation, is that irreversible changes may be produced in some of the proteins when the *p*H of the solution falls below $\sim$ 5·5. It may be mentioned here that it has been a general experience that the fractions obtained at *p*H 5 contain more inhomogeneous heavy material than those gained at *p*H 7.

In Figs. 29 and 30 some diagrams are given which show the amount of the various components precipitated at different ammonium sulphate concentrations from calf serum. It is seen that the precipitation of the fetuin is highly dependent upon the *p*H of the solution and its temperature, while these factors are of less importance for the normal globulin. If, however, the order of fractionation is made thus, $g_1^7 \rightarrow g_1^5 \rightarrow g_2^7 \rightarrow g_2^5 \rightarrow$ *etc.*, there is a tendency for the globulin to be deposited at *p*H 7 and for the fetuin and the 20-component to come down at *p*H 5 (see also Fig. 28, p. 103). It may also be concluded from Fig. 30 that if the precipitations at *p*H 7 are carried out at low temperature (+ 2° to 4° C), while those at *p*H 5 are made at room temperature, a better separation of the globulin may be achieved. It would perhaps also be an advantage to postpone the precipitation at *p*H 5 to the concentration '0·40 Am_2SO_4'; the order should then be $g_1^7 \rightarrow g_2^7 \rightarrow g_3^7 \rightarrow g_3^5 \rightarrow$ *etc.*

It has been observed that the *ga*- and sometimes also some of the *a*-precipitates were very difficult to centrifuge down in the ordinary laboratory centrifuges, and in those cases where they did sediment, the precipitates would not pack tightly. This is undoubtedly due to the presence of '*X*-protein' in these fractions. With human serum the corresponding precipitates generally rose to the top of the centrifuge tube. This is in accordance with the fact that it has not yet been possible to demonstrate any 'density effect' with sera other than human.

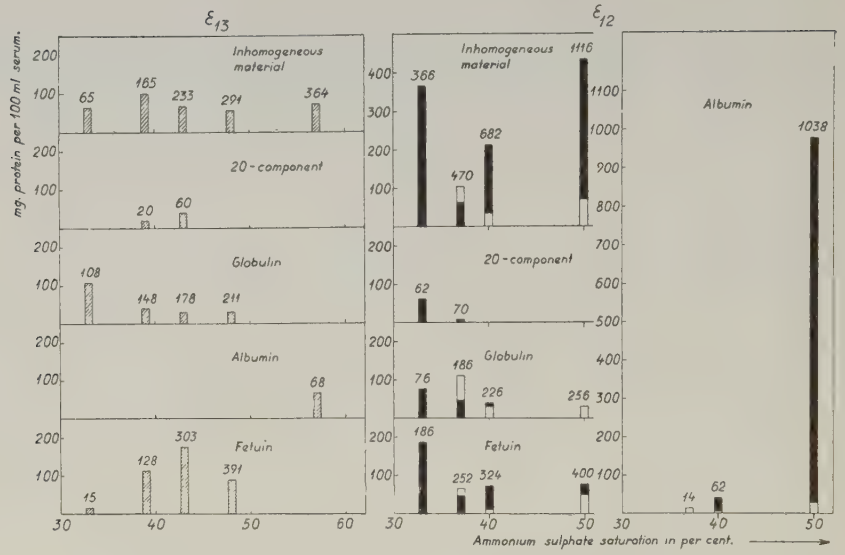

FIG. 29. Amounts of various components precipitated from the same calf serum at a *p*H of about 6, ε_{13}, and at alternating *p*H, ε_{12}.

The height of a rectangle gives the amount of the component precipitated per 100 ml. serum at a given ammonium sulphate concentration, while the figure above the rectangle show the total amount of the component precipitated from the beginning of the experiment. For the fractions from ε_{12}, ε_{16} and ε_{17}, the black areas correspond to *p*H 5 and the white areas to *p*H 7. For a given ammonium sulphate concentration the order of the fraction is indicated in the following way. If the first precipitate has been obtained at *p*H 5 and the second at *p*H 7, then the white rectangle has been placed on top of the black rectangle, and *vice versa*.

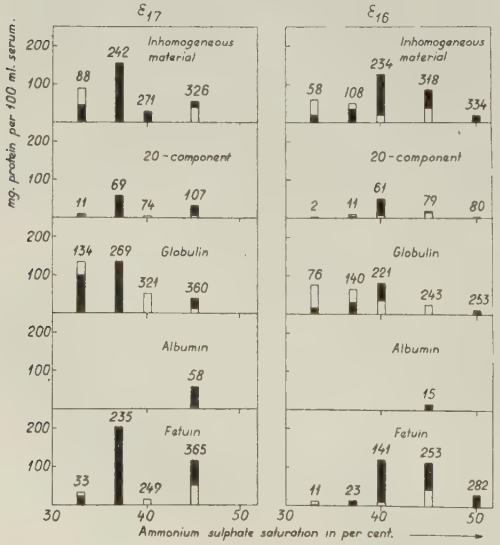

FIG. 30. Amounts of various components precipitated from the same calf serum at alternating *p*H and at different temperatures.

ε_{17} at room temperature (+18° C), ε_{16} in the cold room (+2° to 4° C).

As, however, in other sera too the globulin peak augments with increasing serum concentration less than is to be expected from the total serum concentration, while the opposite is the case for the 'albumin' peak, it must be assumed that the globulin combines in these cases also with some of the other serum constituents to form an *X*-protein. The 'ultracentrifugal' difference between the human '*X*-protein' and those from the other sera should therefore be that the specific volume of the human '*X*-protein' is ~ 1, while the other *X*-proteins should have $1 > V > 0{\cdot}75$, or in any case that $M(1 - V\varrho)/f$ for the *X*-proteins, other than human, should be of the same order of magnitude as for the serum albumins. Attempts have also been made to isolate an *X*-protein from sera, other than human, in a way similar to that described on page 30, but so far in vain. (There is some indication that chicken serum may show a 'density effect' in the ultracentrifuge, and an '*X*-protein' may therefore be prepared from chicken serum in a similar way as from human serum.)

There is another difference between the human *ga*-fractions (and *X*-proteins) and the *ga*-fractions from the other sera studied. The human *ga*-fractions have been found mainly to consist of β-globulin, while the *ga*-fraction from cow besides the albumin (66 per cent) contained 22 per cent α-globulin, 6 per cent β-globulin and 6 per cent γ-globulin (*cf.* Fig. 24, p. 64). According to the method for preparing globoglycoid, this substance is undoubtedly the main component in the *ga*-fraction (sometimes contaminated with serum albumin). Svensson (1941) has found that the globoglycoid from pig serum consisted mainly of α-globulin. From the investigations of Cohn *et al.* (1940, a) it seems most probable that the α-globulin dominates in the *ga*-fraction from horse too. If, however, the globoglycoid is considered as mainly consisting of the '*X*-protein' which is an albumin-globulin-lipid-carbohydrate complex (*cf.* p. 32) it does not seem unreasonable that this compound from horse serum after several re-fractionations could yield crystalbumin (*cf.* Rimington and van den Ende, 1940, and Svensson *l. c.*). This also offers a fair explanation for the finding of Gralén and Pedersen (*cf.* UC p. 374 or UZ p. 338) that their purest horse serum albumin obtained by fractional crystallization with ammonium sulphate always contained 10—15 per cent of molecules with $s_{20} \sim 7$. One of the solutions in question which showed two components in the centrifuge, also contained two components in electrophoresis (Tiselius, unpublished, *cf.* Gralén and Pedersen, *l. c.*), migrat-

ing as albumin and β-globulin. Before, however, the whole problem of the α- and β-globulin and their connection with the albumins, globulins, lipids, carbohydrates, *etc.*, can be definitely solved, much more experimental material must be collected.

The albumin fractions were only occasionally investigated in the ultracentrifuge. They often contained considerable amounts of inhomogeneous heavy material, probably due to contamination with α- and β-globulins. The last albumin fraction generally consisted of two components, one sedimenting as albumin and the other with $s_{20} \sim 3{\cdot}5$. It seems, however, that the ultracentrifuge is not quite as efficient in following the fractionation of the albumins as with the globulins.

In some cases the P/N ratio has been determined for the different fractions. It was found that the P/N ratio was much higher for the *ga*-fractions than for any other When the albumin appeared to be pure, P/N was low, while it was higher in cases where contamination with α- or β-globulin was to be expected, for instance in the most soluble albumin fraction.

From the present investigation it seems likely that all the serum globulins so far used in, for example, solubility studies have been mixtures of ultracentrifugally different components, or, in the most favourable cases, of one component and its first degradation product. The globulin fractions obtained at a concentration of about '0·4 Am_2SO_4' and upwards have, at all events, been mixtures of globulin, the 20-components, some inhomogeneous material and varying amounts of the '*X*-protein'. Many of the earlier studied 'crystalline serum albumins' have undoubtedly been mixtures of albumins with '*X*-proteins' (α- and β-globulins). Such solutions containing *X*-proteins may perhaps be classified in the manner of Sørensen (1930) as 'reversibly dissociable component systems', whereas this classification does not seem correct for the least soluble globulins. It has also been shown by Grönwall (1942), by means of solubility studies, that the isolated euglobulin and pseudoglobulin does not represent such a system. From the investigations of Rimington and van den Ende (1940) it seems that the cryst-albumin of Hewitt (1936, 1937) might be prepared as a pure protein.

It seems reasonable to suppose that many of the troubles connected with the fractionation of the serum proteins are due to the presence of varying amounts of the '*X*-protein'. The great instability of the '*X*-protein' complex may also be responsible for the irreversible changes and denaturation easily produced in many serum fractions.

The predominating position of fetuin in the globulin fraction of fœtal serum was first demonstrated in the blood from sheep's fœtus and then in that from the cow (Pedersen, 1944, a). It was considered of some interest to determine whether the presence of fetuin was of a more general nature, or whether it was present in a few species only. The next serum investigated was obtained from the umbilical blood at the birth of an infant. In this case the amount of fetuin was found to be very low.

From earlier investigations (for references see, for instance, Kuttner and Ratner, 1923), it is known that the placenta of the different animals show different permeabilities towards antibodies. Thus placental transmission of immune substances does not occur in goats and cows, but the antibodies are transferred to the progeny by means of the colostrum. In rodents and man, placental transmission has been demonstrated by many different observers. In the case of ruminants, the placenta consists of three cell layers, whereas the maternal blood is separated from the fœtal by only a single cell layer in *Rodentia* and man.

In order to establish whether the amount of fetuin in the fœtal serum was in some way connected with the structure of the placenta, two more sera were investigated. In fœtal serum from rabbit, it was found that the amount of fetuin was low, although higher than in man. On the other hand, the amount of fetuin in foal serum was found to be of the same order of magnitude as in calf serum (Pedersen, 1944, b).

From Table 47 it is seen that there is a very distinct difference in the relative amounts of fetuin and of globulin between the ruminants on the one side and the rodents and man on the other. In the latter case, where antibodies are known to be transferred from the mother to the fœtus, the serum contains comparatively large amounts of globulin and small amounts of fetuin. In the former case, where the antibodies do not appear in the serum of the new-born animal until it has received colostrum, the relative globulin concentration is very low and that of the fetuin high.

It is still an open question whether or not fetuin is generally present in embryonic serum. It is of interest to note, however, that Svedberg and Andersson (private communication) several years ago, in an unpublished investigation, found that no component with $s_{20} \sim 7$ was present in serum from chicken embryo after 11—15 days incuba-

TABLE 47. APPROXIMATE DISTRIBUTION OF THE VARIOUS PROTEIN COMPONENTS IN SERA FROM FŒTUS AND NEW-BORN ANIMALS, ACCORDING TO PREPARATION EXPERIMENTS.

Class	Serum	Fetuin		'Albumin'		Globulin		20-comp.		Inhomogeneous material			
										From sed. diagram		Not in sed. diagram	
		In total glob.	In total prot.	glob.	prot.	glob.	prot.	glob.	prot.	glob.	prot.	glob.	prot.
		%	%	%	%	%	%	%	%	%	%	%	%
Ruminants	Cow's fœtus	68	28		44	2	1	6	2	1	1	23	24
	Calf	36				18		8		5		33	
	Foal	29	3	25	28	7	1	5	1	9	6	25	61
	Sheep's fœtus	48	21		39	1	1	2	1	10	5	39	33
Rodents	Rabbit's fœtus	6	2		33	39	8	2		11	2	42	53
Man	Umbilical blood	2	1	3	48	54	20	9	3			32	28

tion. The 'albumin' peak in the sedimentation diagram, however, was very asymmetrical and gave comparatively low values for the sedimentation constant. The low value for s_{20} in this case may perhaps be explained by the possible presence of fetuin in the embryonic serum. After 18 days of incubation the globulin amounted to 22 per cent of the protein, and the 'albumin' peak had become more symmetrical.

A question of great interest is why the fetuin is present in fœtal serum. What is its function in these sera? Since the fetuin concentration shows its maximum value in the fœtus, its presence may in some way be associated with the period when the greatest building and development of the animal take place. It may perhaps be needed for the formation of certain parts of the tissue.

Another explanation would be that fetuin is a precursor of one of the proteins in serum, for example the γ-globulin, or perhaps of another component in the 'X-protein' complex. (It seems by no means excluded that this lipo-protein may contain, besides the albumin and the γ-globulin, another, rather unstable, protein.) In order to determine whether the fetuin might be a precursor of some of these proteins, amino acid analyses of fetuin and the other serum proteins have been

planned. The analyses are to be carried out by methods at present being developed at this Institute by Professor Tiselius and his collaborators (for a preliminary report of the method and for further references, see Tiselius, 1944).

In connection with the presence of fetuin in the serum from the fœtus and the new-born animal, it is interesting to note that there seems to exist a special fœtal haemoglobin with properties different from those of the normal adult haemoglobin (for references, see Andersch *et al.*, 1944, and Wyman *et al.*, 1944). These two proteins cannot be distinguished spectrophotometrically, but they differ in their affinity for oxygen and carbon dioxide, in crystalline form, in amino acid content, and in immunological specificity. G. S. Adair found that maternal and fœtal haemoglobin from sheep could be distinguished easily (unpublished work, *cf.* Tiselius, 1940, a, b). Recently Wyman *et al.* (1944) have shown that embryonic carbonylhaemoglobin from cow in strong phosphate buffers at *p*H 6·8 is more than 6 times as soluble as that from the adult. Andersch *et al.* (1944), studying the sedimentation and electrophoresis of haemoglobin from infants at different ages, from the umbilical blood and from adults, have demonstrated large differences in the sedimentation constants of the haemoglobins ($s_{20} = 2{\cdot}5_5$ in a 5 days infant and 4·75 in an adult) and in their electrophoretic mobilities. It is thus evident that in the case of the respiratory proteins also, great differences exist between their properties in the embryonic state and in the adult animal.

Chapter III.

MEASUREMENTS ON INDIVIDUAL SERUM PROTEINS.

1. HISTORICAL.

The serum proteins were among the first substances investigated in the ultracentrifuge by Svedberg and his pupils (Svedberg and Sjögren, 1928, 1930). A review of the works published before the beginning of 1939 has been given by the author (UC p. 372 ff. and p. 401 ff. or UZ p. 337 ff. and p. 364 ff.). The result of the earlier investigations may be summarized as follows.

The serum albumin was stable within the range $3{\cdot}5 < p\mathrm{H} < 11{\cdot}5$ and has a molecular weight of $\sim 70{,}000$ and $s_{20} \sim 4{\cdot}5$. No definite differences between the sedimentation properties of serum albumins from different mammals had so far been observed. According to a preliminary investigation by Svedberg and Andersson (1938) it seemed likely that the serum albumin had the same size in most of the vertebrates. In the lowest class, *Cyclostomata*, two components with $s_{20} = 3{\cdot}5$ and $s_{20} = 12$ predominated in the serum from *Lampetra fluviatilis*, according to the same authors.

From the investigations of Svedberg and Sjögren, *l. c.*, and von Mutzenbecher (1933, c) it appeared that the horse serum globulin was stable at least within the range $5 < p\mathrm{H} < 9$ and perhaps within $4 < p\mathrm{H} < 11$. As a mean value for the sedimentation constant at infinite dilution was given $s^0_{20} = 7{\cdot}1$. For human γ-globulin s_{20} was perhaps somewhat higher (Kabat, 1939). The molecular weights of these globulins were 160,000 to 176,000. According to a private communication, Svedberg and Andersson found several years ago that while the relative amount of the globulin component from the vertebrates varied in an irregular way from species to species, its sedimentation constant showed a more definite trend, in so far as the lower the class of the animal the higher was s_{20}. The higher vertebrates gave $s_{20} \sim 7$, whereas for the lower ones the values were about 8 to 9.

The normal 20-component had not been isolated and no definite values could therefore be given. Kabat (1939) had prepared anti-pneumococcus horse serum globulin and found $s^0_{20} = 19{\cdot}8$. By averaging the diffusion constants for horse, cow and pig immune globulin he arrived at the values $D_{20} = 1{\cdot}71$ and $M = 990{,}000$. For the normal rapidly sedimenting globulin components Svedberg and Andersson (1938 and unpublished) found a characteristic variation with the position of the animal in the classification system. The lower the class of the species was the lower was also s_{20} for the '20-component' but the higher was its concentration. Thus for *Cyclostomata* a component with $s_{20} \sim 12$ (and $M \sim 360{,}000$) predominated. For *Pisces* s_{20} has increased to ~ 16, while for the mammals it was about 18 to 20 (and probably $M \sim 0{\cdot}5$ to $1{\cdot}10^6$).

After 1939 some papers have appeared dealing with the sedimentation properties of serum proteins. Pappenheimer *et al.* (1940) studied a purified diphteria antitoxic horse pseudoglobulin in the ultracentrifuge and in diffusion. They found $s_{20} = 7{\cdot}2$, $D_{20} = 4{\cdot}4$ and, assuming $V = 0{\cdot}736$, $M = 150{,}000$. Kekwick (1940) prepared normal human γ-globulin and globulins from various cases of myeloma. At a concentration corresponding to $\Delta n = 150$ he found values for s_{20} varying between 6·60 and 6·81, in fair agreement with earlier measurements.

From E. J. Cohn's laboratory several measurements on purified serum proteins have been reported. Unfortunately, however, all the sedimentation constants have been given for a protein concentration of 1 per cent which makes a comparison much more difficult. For horse γ-globulin, Cohn *et al.* (1940, a) found $s^1_{2\,0}\% = 6{\cdot}2$ and they estimated $s^0_{20} = 6{\cdot}5$, a very low value compared with those earlier given for horse serum globulin. The β-globulin was found to consist mainly of a component with $s^1_{2\,0}\% = 6{\cdot}3$ and in addition a few per cent of slow-moving components. At higher protein concentration the relative amount of the slow-sedimenting component increased. Their 'α-globulin' was still more complicated, with three components, having $s^1_{2\,0}\% = 17$, 6·8 and 3·6, respectively.

McMeekin (1940) has reported a few runs on some of his crystalline horse serum albumins. For his last prepared carbohydrate-containing albumin he found $s^1_{2\,0}\% = 3{\cdot}6_6$, while the earlier prepared (McMeekin 1939) carbohydrate-free albumin gave $s^1_{2\,0}\% = 3{\cdot}8_5$. He considers the difference between the values obtained by single determinations to be within the experimental errors and concludes: 'Since the

carbohydrate-free albumin gives a value of 4·1 when extrapolated to infinite dilution, we have tentatively assigned this value to both preparations'.

Cohn *et al.* (1940, b) also prepared serum proteins by an ethanol-water method. In the bovine γ-globulin they found a main component with $s^1_{20}\% = 6{\cdot}4$ and a small amount with $s^1_{20}\% = 17$. Their bovine albumin gave $s^1_{20}\% = 4{\cdot}0$. For another sample of bovine serum albumin Lauffer (*cf.* Putnam *et al.* 1943) found $s^1_{20}\% = 4{\cdot}06 \pm 0{\cdot}27$.

For human fibrinogen Holmberg (1944, a) has found $s_{20} = 8{\cdot}5$, $D_{20} = 1{\cdot}1$ and $M \sim 700{,}000$.

In a recent publication Cohn *et al.* (1944, Table I, p. 422)[1] have given some physical constants for a few of the human plasma proteins in dilute solutions.

TABLE 48. PHYSICAL CONSTANTS OF SOME PLASMA PROTEINS ACCORDING TO COHN *et al.* (1944).

Protein	s_{20}	D_{20}	M
Serum albumin	4·6 [a]	6·1 [a]	69,000 [b]
Serum γ-globulin	7·1 [c]	3·8 [c]	156,000 [c]
Fibrinogen	7 [a]		500,000 [d]

a. Unpublished measurements of Oncley, J. L.

b. Calculated from sedimentation and diffusion constants and from osmotic pressure measurements.

c. The sedimentation and diffusion constants recorded here are those quoted by Svedberg and Pedersen (1940) for total, electrophoretically isolated, human γ-globulin. There are indications that this sedimentation constant and the molecular weight, given as 176,000, are somewhat larger than those for our γ-globulin Fraction II, which represents only a part of the total γ-globulin of plasma, and probably more of the pseudoglobulin than of the euglobulin. Complete ultracentrifugal and diffusion studies have not yet been carried out on this subfraction, but a value of 156,000 for the molecular weight of such a preparation has been obtained from osmotic pressure measurements by Scatchard, G., Brown, A., and Batchelder, A. C. This value may well vary with the euglobulin/pseudoglobulin ratio of the γ-globulin fraction, lower values being characteristic of pseudoglobulin.

d. Estimated from measurements of sedimentation constant, viscosity, and double refraction of flow, the latter by J. T. Edsall and I. H. Scheinberg.

[1] The paper of Cohn *et al.* is the first of a series of investigations, all published in the July number (1944) of 'The Journal of Clinical Investigation', under the joint heading: 'Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation'. This publication was not available at Upsala before the middle of January 1945, when the greater part of the present work was already in print.

There is thus a definite difference between Holmberg's and Oncley's values for fibrinogen. Regarding some of the other components, Cohn *et al.* (1944, p. 421) say: 'Fibrinogen, γ-globulin, and a part of the α- and β-globulins, have sedimentation constants near 7 *S*. In addition, there are small amounts of globulins sedimenting with constants between 7 and 18 *S*; and, although the results are not yet conclusive, it would appear that at least a part of the α- and β-globulins sediment, under certain conditions, with a rate close to that of albumin.'

2. GENERAL.

The molecular weight of a protein may be calculated from s^0_{20} and D^0_{20} by means of the Svedberg formula:

$$M = \frac{R\,T \cdot s}{D(1 - V\varrho)}$$

V is determined from density measurements (UC p. 57 ff. or UZ p. 52 ff.) or may be estimated from earlier experiments on kindred proteins.

As a rule, the sedimentation constant varies quite considerably with concentration and it is necessary to determine s^0_{20} by extrapolation of the line of regression in the $s - c$, or in some cases the $s - s \cdot c$, graph to $c = 0$. In order to provide a measure for the fitting of the various lines of regression for the different proteins, the coefficient of correlation has also been given.

The diffusion measurements have been carried out according to Lamm (1937, 1940) and in his slide cell. The diffusion constant usually varies but little with concentration, and if an average value for D_{20}, obtained from experiments carried out with below 1 per cent protein, is used instead of D^0_{20}, the error involved is small.

The partial specific volume, V, does not change much from protein to protein. For the albumins V_{20} is $\sim 0{\cdot}75$, for the γ-globulins $V_{20} \sim 0{\cdot}73$, while for fetuin $V_{20} \sim 0{\cdot}70$. Usually V is determined from solutions having a concentration of a few per cent, and very little is known about the variation of V with the protein concentration and the composition of the buffer solution. It may be supposed, however, that such variations are rather small (*cf.* UC p. 51—52, 61 or UZ p. 47—48, 55—56). The molecular weight calculated by means of Svedberg's formula gives the weight of the *unhydrated* particle.

In certain instances where, for instance, very little of the substance is available, it may not be possible to determine V in the direct way. If, however, the density of the particle, ϱ_P, may be estimated from sedimentation measurements in solutions having different densities, the weight of the *hydrated* molecule may be calculated by substituting $1/\varrho_P$ for V in Svedberg's formula. Generally the molecular weight calculated by the two methods are different. Only in the case of unhydrated molecules do the two methods give the same result.

During the present investigation it has been found that many of the protein solutions contain considerable quantities of heavy inhomogeneous material, which sediments to the bottom of the centrifuge cell before the measurements were started. This means that the concentration of the sedimenting substance is now less than that in the original solution and that s_{20} calculated from the diagrams only refers to part of the material. When the same solution is investigated in diffusion, the whole of the material is represented in the diffusion curve. In such a case the two diffusion constants, D_A and D_m[1], differ from one another. Spinning of the solution in the air-driven centrifuge at 27,000 R.P.M. for some hours generally removes most of the heavy material and brings the values of D_A and D_m closer together, as will be shown later. It is therefore very important to remove such heavy material before studying a protein solution in diffusion.

Sometimes the protein solutions have been contaminated with small amounts of the 20-component, but the solution has nevertheless been run in diffusion. In such cases the percentage of this component has been determined from the sedimentation diagram and based on this, and an estimated diffusion constant for the 20-component; the theoretical diffusion curves for this component have been constructed for the various exposures and subtracted from the experimental diffusion curve before this is used for the final calculation of D_A and D_m.

3. EXAMPLES.

In the present work the main interest was directed towards finding suitable methods for preparing pure and well defined serum proteins. This object was, however, not reached, although valuable information was gained which may eventually lead to its attainment. From time

[1] D_A and D_m mean that the diffusion constants are obtained by the 'area' or the 'moment' method of calculation, respectively (*cf.* Gralén 1941, 1944).

to time during the progress of the work, fractions were obtained which appeared comparatively pure in the ultracentrifuge. They were then further investigated in sedimentation and diffusion, and the results are given below. Under the circumstances, the values obtained must not be considered as final, but only as preliminary.

A. Fetuins.

Some of the results obtained with fetuin have already been published (Pedersen, 1944, a). As, however, the extrapolation of s_{20} was not formerly made in the same way as in this publication, a recalculation of the line of regression has been carried out after the addition of some more recent values for s_{20} (Table 49).

Table 49. Sedimentation Constants for Fetuin in Different Fractions from Calf Serum.

Fraction	Δn for Fetuin	s_{20}	Fraction	Δn for Fetuin	s_{20}	Fraction	Δn for Fetuin	s_{20}
$g_1\,\varepsilon_1$	223	2·87	$g_3^5\,\varepsilon_{12}$	137	3·60	$(g_3^5\,\varepsilon_{14})_3^7$	253	2·40
$g_1\,\varepsilon_1$	245	2·61	$g_2\,\varepsilon_{13}$	358	2·46	$(g_3^5\,\varepsilon_{14})_3^5$	255	2·64
$g_{1am}\,\varepsilon_1$	234	2·82	$g_3\,\varepsilon_{13}$	325	2·70	$g_1^5\,\varepsilon_{15}$	44	3·18
$g_{1amA}\,\varepsilon_1$	60	3·23	$(g_3\,\varepsilon_{13})_1^5$	20	3·16	$g_2^5\,\varepsilon_{15}$	92	2·94
do.	111	3·06	do.	37	3·18	$g_2^5\,\varepsilon_{16}$	44	3·18
do.	222	2·76	do.	74	3·11	$g_3^7\,\varepsilon_{16}$	31	3·31
do.	539	2·16	do.	169	2·91	$g_3^5\,\varepsilon_{16}$	227	2·88
$g_{1b}\,\varepsilon_1$	360	2·61	do.	257	2·78	$g_2^5\,\varepsilon_{17}$	223	2·88
$g_2\,\varepsilon_1$	185	2·92	do.	282	2·64	$e_4\,\varepsilon_{18A}$	159	3·05
$g_1\,\varepsilon_4$	345	2·78	do.	406	2·54	$e_5\,\varepsilon_{18A}$	107	2·98
$g_1\,\varepsilon_5$	282	2·96	$(g_3\,\varepsilon_{13})_2^5$	228	2·98	$e_4\,\varepsilon_{18B}$	89	3·22
$g_1\,\varepsilon_8$	333	2·55	$(g_3\,\varepsilon_{13})_3^7$	135	3·19	$e_5\,\varepsilon_{18B}$	206	2·97
$g_2\,\varepsilon_9$	190	2·66	$g_1^5\,\varepsilon_{14}$	103	2·99	$e_6\,\varepsilon_{18B}$	125	3·04
$g_1\,\varepsilon_{10}$	46	3·01	$g_2^5\,\varepsilon_{14}$	170	3·01	$e_4\,\varepsilon_{19A}$	218	2·87
$g_2\,\varepsilon_{10}$	156	2·85	$(g_2^5\,\varepsilon_{14})_2^5$	164	2·80	$e_5\,\varepsilon_{19A}$	85	3·17
$g_1^5\,\varepsilon_{12}$	109	3·22	$(g_2^5\,\varepsilon_{14})_3^7$	229	2·67	$e_4\,\varepsilon_{19B}$	243	2·84
$g_2^5\,\varepsilon_{12}$	161	3·15	$g_2^7\,\varepsilon_{14}$	27	3·52	$e_5\,\varepsilon_{19B}$	121	2·88
$g_2^7\,\varepsilon_{12}$	83	2·83	$g_3^5\,\varepsilon_{14}$	214	2·96			
$g_3^7\,\varepsilon_{12}$	75	3·11	$(g_3^5\,\varepsilon_{14})_1^5$	73	3·24			

For the line of regression and the correlation coefficient the following values were obtained:

$$s_{20} = 3{\cdot}28 - 0{\cdot}00200 \cdot \Delta n \qquad r = 0{\cdot}86.$$

The new value for s_{20} agreed excellently with the old, $s_{20} = 3{\cdot}29$, but the correlation was less good for the fetuin than for the other serum proteins. Only the values obtained for fetuin from calf serum have been included in Table 49, since it appeared that there might be some difference between the values of s_{20} for the calf and those for the fœtus.

The following values were taken from the different fractions from fœtal sera containing fetuin.

From Table 31 (p. 112),

Δn (from diagram) =	153	304	364	442
s_{20} =	3·05	2·51	2·56	2·50

From Table 32—33 (pp. 112—113),

Δn (from diagram) =	290	346	395
s_{20} =	2·79	2·58	2·60

From Table 34 (p. 113),

Δn (from diagram) =	118	268	297	368
s_{20} =	3·03	2·66	2·56	2·47

From different dilutions of $G_{III}\zeta_4$,

Δn (from diagram) =	10	16	167
s_{20} =	2·94	3·11	2·76

The equation for the line of regression was determined, and the coefficient of correlation was calculated both for the s—Δn and the s—$s \cdot \Delta n$ graph. These were, respectively:

$$s_{20} = 3{\cdot}09 - 0{\cdot}00145 \cdot \Delta n \qquad r = 0{\cdot}89$$
$$s_{20} = 3{\cdot}10 - 0{\cdot}00057 \cdot s \cdot \Delta n \qquad r = 0{\cdot}86$$

The fitting of the line is thus a little better in the former case; it is also seen that the correlation is slightly better for fetuin from the fœtus, than for that from the calf.

Comparison of the values of s_{20} for fetuin from calf and that from cow's fœtus shows some difference. In order to draw any definite conclusion from the difference it seems necessary to have many more experimental data on fœtal fetuin. If, nevertheless, an analysis of the

variance is carried out, it shows that there is a probability of 95 per cent that the sedimentation properties are different for fetuin from calf and fetuin from fœtus.

Several diffusion experiments have been carried out on fetuin prepared both from calf sera and from fœtal serum. The first experiment on fetuin from calf has already been published (Pedersen, 1944). The run was made on the middle fraction from a three-hour separation of $(g_3\,\varepsilon_{13})_1^5$ in the air-driven centrifuge at 27,000 R.P.M. From the sedimentation diagram it was found that 89 per cent was fetuin and 11 per cent the 20-component. The diffusion constants were ($\Delta n = 138$) $D_A = 4{\cdot}65$ and $D_m = 5{\cdot}08$. After the curves had been corrected for the presence of the 20-component (tentative value $D_{20} = 1{\cdot}65$), D_{20} for the fetuin was found to be $D_A = 5{\cdot}68$ and $D_m = 5{\cdot}51$. The same solution was also run at a concentration corresponding to $\Delta n = 250$. The original values were then $D_A = 4{\cdot}48$ and $D_m = 5{\cdot}10$; after correction for the presence of the 20-component, D_{20} for the fetuin was found to be $D_A = 5{\cdot}35$ and $D_m = 5{\cdot}72$.

Several diffusion runs were carried out on fetuin from fœtal serum with the following result:

No.	Fetuin solution	Δn in experiment	D_A	D_m
1	$G_{IIIA}\,\zeta_4$	256	4·21	4·61
2		128	4·62	4·91
3		65	4·06	4·27
4		31	4·37	4·13
5	$G_{IIIB}\,\zeta_4$	102	4·89	4·69

The first series of experiments was carried out in order to determine the variation of the diffusion constant with concentration for fetuin. The values given above showed, however, such a fluctuation that they could not be used for the purpose. The experiments which appeared to be the most reliable were Nos. 2 and 5. In No. 2 all the curves were corrected for the presence of a component with $D_{20} = 1{\cdot}65$ in a relative concentration of 2 per cent. After the correction it gave $D_A = 4{\cdot}85$ and $D_m = 4{\cdot}98$. The curves were all reduced to normal coordinates both before and after the correction for the 20-component. The two average curves were compared with the ideal curve and both were found to be over-normal, more so before the correction than after. In No. 5 the corrections were only made on the maximum heights of the curves and their areas. The corrected value for D_A was 4·96

As the most reasonable value for the diffusion constant of fœtal fetuin we may take $D_{20} = 5{\cdot}0$.

The partial specific volume of fetuin was determined on different preparations. For fetuin from calf a value of $V_{20} = 0{\cdot}714$ was found for $g_{1\text{amA}}\ \varepsilon_1$. For fetuin from cow's fœtus two rather different values were obtained. For $G_{\text{IIIA}}\,\zeta_4$ was found $V_{20} = 0{\cdot}692$, while a less fractionated fetuin $G\,\zeta_8$ gave $V_{20} = 0{\cdot}712$. For the nitrogen and phos-

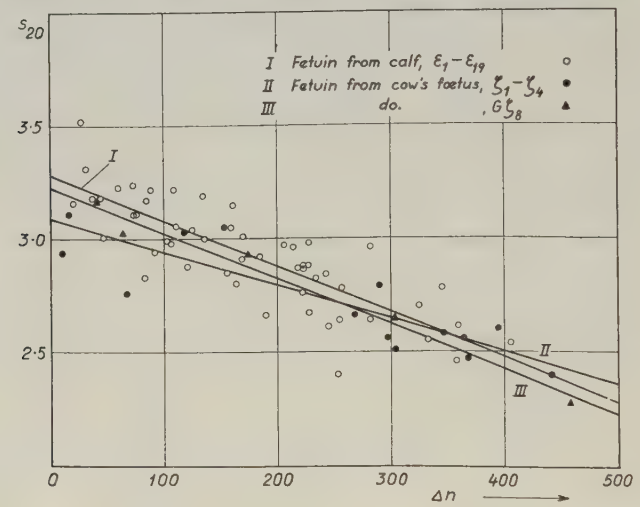

FIG. 31. Variation of s_{20} with concentration of fetuin, as measured from the sedimentation diagram.

phorus contents of the two preparations were found: for $G_{\text{IIIA}}\,\zeta_4$ 12·3 g. N/100 g. protein and 0·188 g. P/100 g. protein, for $G\,\zeta_8$ 13·4 g. N/100 g. protein and 0·101 g. P/100 g. protein. The two materials thus seemed to be rather different. The nitrogen content of the fetuin was rather low for a protein.

For the molecular weights of the fetuin of different origin we find:

For fetuin from calf,

$$s_{20} = 3{\cdot}28,\ D_{20} = 5{\cdot}5 \text{ and } V_{20} = 0{\cdot}714 \text{ give } M = 50{,}600.$$

For fetuin from cow's fœtus,

$$s_{20} = 3{\cdot}09,\ D_{20} = 5{\cdot}0 \text{ and } V_{20} = 0{\cdot}692 \text{ give } M = 48{,}700.$$

If $V_{20} = 0{\cdot}692$ is erroneous and 0·712 is the correct value for V, we find $M = 52{,}000$.

Quite recently a series of measurements of s_{20} were carried out on the fraction from fœtal fetuin, $G\,\zeta_8$, which had given $V_{20} = 0{\cdot}712$, as compared with $V_{20} = 0{\cdot}714$ for fetuin from the calf and $V_{20} = 0{\cdot}692$ from the fœtus. The new values were:

Δn (from diagram) =	40	63	173	303	458
s_{20} =	3·17	3·03	2·93	2·65	2·28

which gives

$$s_{20} = 3{\cdot}23 - 0{\cdot}00200 \cdot \Delta n \qquad r = 0{\cdot}99$$

This fœtal fetuin thus greatly resembles the fetuin from the calf, as regards both s_{20} and V_{20}. Diffusion measurements have not yet been made, but it seems reasonable to suppose that this new sample of fetuin will give a D_{20} close to that for the fetuin from calf, since the coefficient of regression is the same in the two cases (*cf.* also Fig. 31).

B. Serum Albumins.

a. Human Serum Albumins.

In normal human serum albumin the following values were found for the sedimentation constant. (See Fig. 32.)

From Table 36 (p. 118)

Δn (from diagram) =	166	267	299
s_{20} =	4·17	4·02	4·03

From Table 37 (p. 119)

Δn (from diagram) =	126	344	426
s_{20} =	4·40	3·95	3·81

For different dilutions of $a_2\alpha_{12}$

Δn (from diagram) =	45	90	130
s_{20} =	4·65	4·52	4·41

The expressions for the line of regression and the coefficient of correlation were, respectively,

$$s_{20} = 4{\cdot}67 - 0{\cdot}0022 \cdot \Delta n \text{ and } r = 0{\cdot}97.$$

Two diffusion runs were made; the first at a concentration of 0·5 per cent and the second at 0·25 per cent. The first gave $D_A = 6{\cdot}00$ and $D_m = 5{\cdot}31$, while the second gave $D_A = 5{\cdot}85$ and $D_m = 5{\cdot}53$. From $s_{20} = 4{\cdot}67$, $D_{20} = 5{\cdot}93$ and $V_{20} = 0{\cdot}736$ we obtain $M = 72{,}300$.

b. Serum Albumins from Cow's Fœtus and from Calf.

The albumin fractions often appeared rather inhomogeneous and contained much heavy material. As, however, the fœtal serum from cow gave albumin fractions which seemed to be somewhat more homogeneous, the fœtal albumin was investigated a little further. The following sedimentation constants were considered representative for this albumin. (See Fig. 32.)

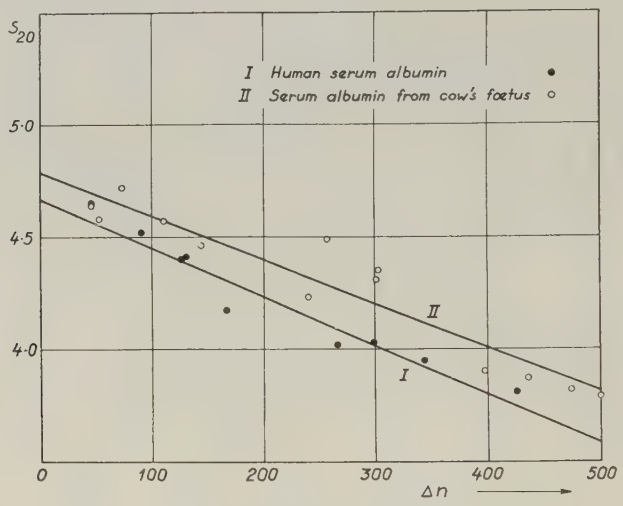

FIG. 32. Variation of s_{20} with concentration of serum albumin, as measured from the sedimentation diagram.

From Table 31 (p. 112)

Δn (from diagram) =	240	474	
s_{20} =	4·23	3·82	

From Table 32 (p. 112)

Δn (from diagram) =	257	436	501
s_{20} =	4·49	3·87	3·79

From Table 34 (p. 113)

Δn (from diagram) =	301	303	398
s_{20} =	4·31	4·35	3·90

Finally five dilutions $a_2\zeta_3$ were run:

Δn (from diagram) =	45	52	73	110	144
s_{20} =	4·64	4·58	4·72	4·57	4·46

The expressions for the line of regression, and the coefficient of correlation were, respectively,

$$s_{20} = 4{\cdot}79 - 0{\cdot}00194 \cdot \Delta n \qquad r = 0{\cdot}94.$$

A diffusion experiment was made at a concentration corresponding to $\Delta n = 115$ on a solution of $a_2\zeta_3$ which had been run at 27,000 R.P.M. for 3 hours. For D_{20} was found $D_A = 6{\cdot}49$ and $D_m = 6{\cdot}21$.

From $s_{20} = 4{\cdot}79$, $D_{20} = 6{\cdot}49$ and $V_{20} = 0{\cdot}736$ (tentative value) we find $M = 67{,}800$.

One of the albumin fractions, $a_2\varepsilon_{16}$, which contained a large amount of inhomogeneous material, was investigated in diffusion before and after being spun in the air-driven centrifuge at 27,000 R.P.M. for 3 hours. In the former case the diffusion curves were evidently of a composite nature and only the D_A was calculated and found to be 4·41. In the latter case normal diffusion curves were obtained with $D_A = 5{\cdot}78$ and $D_m = 5{\cdot}68$.

C. Serum Globulins.

a. γ-Globulin from Cow.

The first γ-globulin to be examined a little more in detail was the $P_{II}\delta_1$ (p. 70). It was not quite homogeneous in the centrifuge, but contained ~ 5 per cent of fetuin (or '*X*-protein').

Six different dilutions of the solution were run with the following result:

Δn (from diagram) =	92	132	168	269	373	533
s_{20} =	7·16	6·85	6·70	6·31	6·09	5·27

The equation for the line of regression was found to be

$$s_{20} = 7{\cdot}43 - 0{\cdot}00397 \cdot \Delta n$$

and the coefficient of correlation was $r = 0{\cdot}991$. (See Fig. 33.)

Diffusion experiments were carried out at concentrations of both about 1 per cent and 0·5 per cent protein. In the former case the result was $D_A = 3{\cdot}32$ and $D_m = 3{\cdot}52$, and in the latter $D_A = 3{\cdot}74$ and $D_m = 3{\cdot}95$ for a temperature of 20·0° C.

From $s_{20} = 7{\cdot}43$, $D_{20} = 3{\cdot}74$ and $V_{20} = 0{\cdot}732$ (tentative value) we obtain $M = 180{,}000$.

The first globulin fractions, $g_1\delta_7$ and $g_1\delta_8$, from Fractionation Experiment No. 5 (p. 73) were both further examined in sedimentation and diffusion.

For $g_1\delta_7$ the following values were found:

c (g./100 ml.) =	0·77	1.55	3·16	4·63	6·95
s_{20} =	7·27	6·97	7·02	6·32	5·32

The line of regression and the coefficient of correlation were determined both for the $s - c$ and for the $s - s \cdot c$ graph. For the former it was

$$s_{20} = 7{\cdot}61 - 0{\cdot}30 \cdot c\,(= 7{\cdot}61 - 0{\cdot}0025 \cdot \Delta n) \qquad r = 0{\cdot}95$$

and for the latter

$$s_{20} = 7{\cdot}73 - 0{\cdot}055 \cdot s \cdot c \qquad r = 0{\cdot}90$$

The correlation is thus best in the $s - c$ graph.

The diffusion experiments were carried out about a year after the preparation of $g_1\delta_7$ and $g_1\delta_8$. In the meantime part of the inhomogeneous material had deposited in the stock solution and for $g_1\delta_7$, Δn had dropped from 1389 to 1150. At a concentration corresponding to $\Delta n = 115$ the following values were found:

$$D_A = 3{\cdot}62 \text{ and } D_m = 3{\cdot}72.$$

The solution was then spun in the air-driven centrifuge for 3 hours at 27,000 R.P.M., when some more material settled to the bottom of the centrifuge tube. The composition of this material was 51 per cent globulin, 4 per cent 20-comp. and 45 per cent inhomogeneous material. The supernatant was run in diffusion and gave:

$$D_A = 3{\cdot}58 \text{ and } D_m = 3{\cdot}48 \quad (\Delta n = 117).$$

In the supernatant V_{20} was found to be 0·732 at a concentration of 2·75 g. protein/100 ml. solution. For dn/dc a value of 0·00179 was obtained ($\lambda = 546$ mμ).

From $s_{20} = 7{\cdot}61$, $D_{20} = 3{\cdot}58$ and $V_{20} = 0{\cdot}732$ we have $M = 192{,}000$, which seems to be a rather high value.

For $g_1\delta_8$ the following values were found

c (g./100 ml.) =	0·20	0·40	0·79	1·18	1·58	2·36	3·15	5·72	6·29	9·44
s_{20} =	7·38	7·48	7·39	7·22	6·88	6·85	6·79	6·52	5·41	4·13

For the line of regression and the coefficient of correlation were found:

$$s_{20} = 7{\cdot}65 - 0{\cdot}35 \cdot c\,(= 7{\cdot}65 - 0{\cdot}0029 \cdot \Delta n) \qquad r = 0{\cdot}98$$

or

$$s_{20} = 7{\cdot}79 - 0{\cdot}062 \cdot s \cdot c \qquad r = 0{\cdot}89.$$

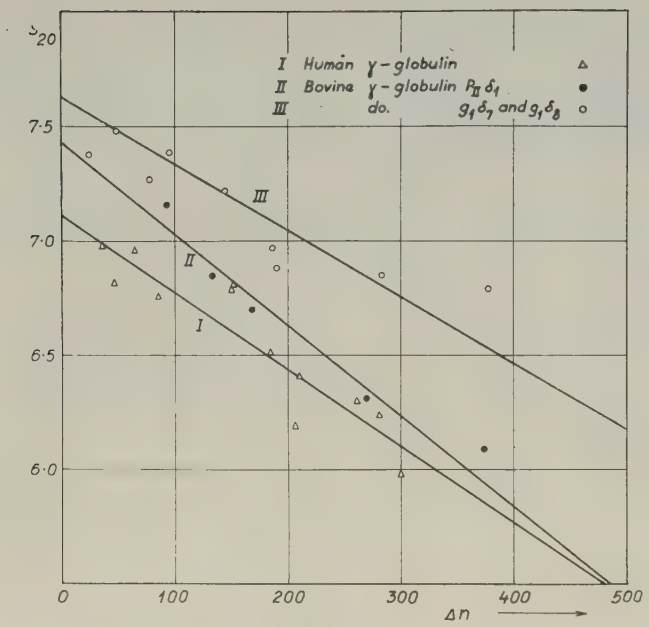

FIG. 33. Variation of s_{20} with concentration of serum globulin, as measured from the sedimentation diagram.

The correlation is thus still better here in the $s - c$ graph than in the $s - s \cdot c$ graph.

On standing, a large part of the inhomogeneous material deposited in the stock solution and its Δn dropped from 1888 to 1300. At a concentration corresponding to $\Delta n = 104$ the following values were found: $D_A = 3{\cdot}92$, $D_m = 4{\cdot}04$.

The deposit from 3 hours spinning at 27,000 R.P.M. was found to consist of 67 per cent normal globulin and 32 per cent inhomogeneous material. The supernatant gave in diffusion $D_A = 3{\cdot}81$ and $D_m = 3{\cdot}73$.

From $s_{20} = 7{\cdot}65$, $D_{20} = 3{\cdot}81$ and $V_{20} = 0{\cdot}732$ (tentative value), we obtain $M = 182{,}000$.

b. Human γ-globulin.

Some normal and pathological human γ-globulins were investigated in diffusion and sedimentation.

For the normal globulin were found, for $g_1^1\alpha_{11}$ (p. 118)

Δn (from diagram) =	151	184	210
s_{20} =	6·81	6·51	6·41

for $g_1^7\alpha_{12}$ (p. 119)

Δn (from diagram) =	36	64	206	261	300
s_{20} =	6·98	6·96	6·19	6·30	5·98

for $g_1^7\alpha_{13}$ (new preparation similar to α_{11}, but not described in this publication)

Δn (from diagram) =	46	85	150	281
s_{20} =	6·82	6·76	6·79	6·24

For the line of regression and the coefficient of correlation the values obtained were:

$$s_{20} = 7{\cdot}12 - 0{\cdot}0034\,\Delta n \qquad r = 0{\cdot}92.$$

$g_1^7\alpha_{11}$ was also run in diffusion after having been spun for 4 hours at 27,000 R.P.M. For a solution with $\Delta n = 108$ the two D_{20} were $D_A = 3{\cdot}98$ and $D_m = 4{\cdot}02$, while a more dilute solution with $\Delta n = 31$ gave $D_A = 4{\cdot}00$ and $D_m = 4{\cdot}01$.

The specific volume was determined on the same solution at a concentration of 2·58 g. protein/100 ml. solution and found to be $V_{20} = 0{\cdot}718$. For dn/dc the very low value of 0·00172 ($\lambda = 546$ mμ) was obtained.

From $s_{20} = 7{\cdot}12$, $D_{20} = 4{\cdot}00$ and $V_{20} = 0{\cdot}718$ we have $M = 153{,}000$.

An anticomplementary pseudoglobulin was investigated for Dr. B. Olhagen, Stockholm, in sedimentation and diffusion. The sedimentation diagram showed that the first preparation investigated contained 12 per cent of a substance with lower sedimentation constant (probably albumin or perhaps some α- or β-globulin). Five different dilutions of the solution were run in the centrifuge and gave

Concentration =	0	0·15 c_0	0·25 c_0	0·5 c_0	0·75 c_0	c_0
s_{20} =	(7.14)	7·09	7·00	6·98	6·74	6·73

Two diffusion experiments gave for a concentration of 0·5 per cent $D_A = 3{\cdot}92$ and $D_m = 3{\cdot}67$, and for 0·7 per cent protein $D_A = 3{\cdot}71$ and $D_m = 3{\cdot}96$. Subtraction of 12 per cent albumin with $D_{20} = 6{\cdot}1$ from the diffusion curves in the first experiment reduced the values to $D_A = 3{\cdot}80$ and $D_m = 3{\cdot}44$.

For the specific volume was found $V_{20} = 0{\cdot}732$. A different anticomplementary pseudoglobulin gave $V_{20} = 0{\cdot}73_0$.

From $s_{20} = 7{\cdot}14$, $D_{20} = 3{\cdot}80$ and $V_{20} = 0{\cdot}732$, we find $M = 170{,}000$.

c. Other Globulins.

An antipneumococcus globulin from rabbit serum was investigated in sedimentation and diffusion for Dr. M. Björneboe, Copenhagen. In

a 2·2 per cent solution the percentage composition, according to the sedimentation diagram, was: 'albumin': 6, globulin: 38, inhomogeneous material in sedimentation diagram: 9, inhomogeneous heavy material: 47. For a 1·1 per cent solution the values were, respectively: 7, 64, 3 and 26. The solution was also run in diffusion and gave $D_A = 3{\cdot}87$ and $D_m = 4{\cdot}45$.

A 2 per cent solution was then run in the air-driven centrifuge at 27,000 R.P.M. for 4 hours. During the run a massive pellet had collected at the bottom of the centrifuge tube. It could be dissolved in 0·2 M-NaCl and it was then run in the ultracentrifuge in a concentration of 0·8 per cent. The composition, according to the ultracentrifuge diagram, was 34 per cent globulin and 66 per cent inhomogeneous heavy material. Diffusion experiments on the supernatant gave $D_A = 3{\cdot}98$ and $D_m = 4{\cdot}04$, which indicated a considerable improvement. It was also investigated in the centrifuge (but not until a month later) at a protein concentration of 1·3 per cent. According to the diagrams the percentage composition was: 'albumin': 7, globulin: 55, inhomogeneous material in the diagrams: 26, and inhomogeneous heavy material: 12.

D. The 20-Components.

Several attempts were made to isolate the 20-component from cow serum by repeated separation, in the air-driven centrifuge, of the heavy component from globulin fractions rich in the 20-component. As more and more of the other globulins were removed with the supernatant from the separations, the 20-component became increasingly difficult to dissolve. It appeared that it was seriously changed (denatured?) during such a purification. It was therefore planned to use albumin solutions as solvents instead of the 0·2 M-NaCl employed in the experiments just described. As, however, very pure albumin solutions were necessary for such a purpose, the experiments had to be postponed for the time being.

Normal human serum contains very variable amounts of the 20-component, but it has been a general experience that as soon as the iso-haemagglutinin titer is high, the amount of the 20-component is also high in the serum, and when the titer is low the amount of the 20-component is low. Preliminary experiments in the separation cell (UC p. 152 or UZ p. 132) with sera belonging to the different blood-groups have shown that the agglutinins sediment at the same rate as

the 20-component. In case of the serum from an individual belonging to the O-group a slight difference was observed between the rate of sedimentation for the α- and the β-agglutinins. This small difference, however, may have been a mere accident, due to the difficulty in the titration when the agglutinin concentration is low. It is also still an open question whether in *normal* human serum the whole 20-component consists of the iso-haemagglutinins.

From an A-serum with a high β-agglutinin titer, the 20-component was isolated and purified by repeated separations in the air-driven centrifuge at 27,000 R.P.M. The final solution contained less than 10 per cent of other ultracentrifugal components. The volume of solution was just sufficient for making the following 5 determinations:

Δn (from diagram) =	18	34	59	95	166
s_{20} =	19·85	19·5	19·35	18·9	18·7

which gives:

$$s_{20} = 19{\cdot}81 - 0{\cdot}0074 \cdot \Delta n \qquad r = 0{\cdot}95$$

Two '20-component' fractions from pathological human sera (Cases I and II from Waldenström (1944, a)) were further investigated in sedimentation and diffusion.

From Case I serum, euglobulin was prepared by a fortyfold dilution of the serum with water. The precipitate $eug_1\beta_7$ was centrifuged sharply down and finally dissolved in 0·2 M-NaCl. The euglobulin was prepared from the other serum (Case II) by adjusting its pH to 6 and dialysing it against a phosphate buffer with the same pH and $\mu = 0{\cdot}02$, the precipitate $eug_1\beta_9$ was centrifuged tightly down and then dissolved in 0·2 M-NaCl. It was re-precipitated twice in the same way and the final product $eug_{1aA}\beta_9$ was separated in the air-driven centrifuge in order to remove as much as possible of the 7-component.

The two fractions were investigated in the ultracentrifuge at 40,000 R.P.M.

For $eug_1\beta_7$ were found:

Relative concentration =	1·000	0·333	0·167	0·084	0·042
s_{20} =	13·29	17·82	17·57	18·73	18·95

From the lower concentrations the ratio between the 'relative concentration' and the Δn for the 20-component peak in the sedimentation

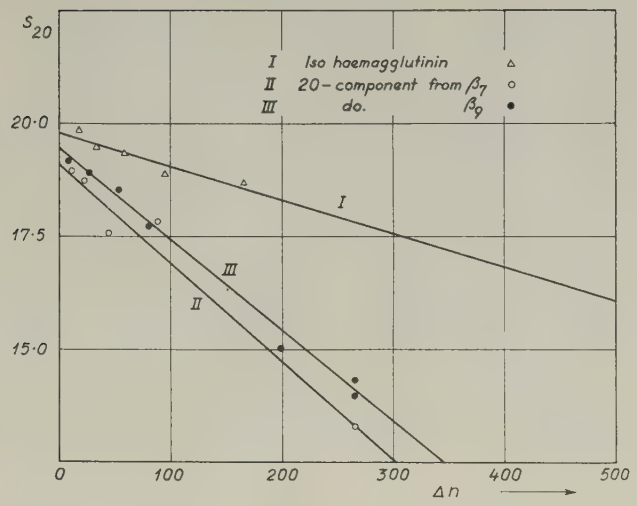

FIG. 34. Variation of s_{20} with concentration of the 20-component, as measured from the sedimentation diagram.

diagram could be obtained. The variation of s_{20} with concentration was found to be (see also Fig. 34)

$$s_{20} = 19{\cdot}14 - 0{\cdot}0219 \cdot \Delta n \qquad r = 0{\cdot}98.$$

For $eug_{1aA}\,\beta_9$ were found:

Relative concentration =	1·00	1·00	0·75	0·30	0·20	0·10	0·07	0·03
s_{20} =	13·97	14·34	15·03	17·74	18·55	18·93	19·30	19·17

$$s_{20} = 19{\cdot}4_8 - 0{\cdot}0218 \cdot \Delta n \qquad r = 0{\cdot}996.$$

The salt concentration of $eug_{1aA}\,\beta_9$ was then increased from 0·2 M-NaCl to 1 M-NaCl and the following values were obtained:

Relative concentration =	1·00	0·7	0·3	0·2
s_{20} =	14·15	15·59	17·37	18·37

$$s_{20} = 19{\cdot}1 - 0{\cdot}0202 \cdot \Delta n \qquad r = 0{\cdot}994.$$

Several diffusion measurements were made on solutions containing the pathological 20-component, but great variations were found in D_{20} probably partly due to the amounts of inhomogeneous material and heavy components present. The following values were obtained:

No.	D_A	D_m	Other components according to centrifuge diagrams	
1	1·63	1·68	7++	24+
2	1·43	1·41	7+	24+
3	1·19	1·21	7—	28++
4	1·49	1·51	4(+) 7(+)	
5	1·25	1·27	7+	inhomogen.

Nos. 1—4 were from Case II, while No. 5 was from Case I. It is somewhat difficult to ascertain what value should be allotted to D_{20}, but it seems most reasonable to assume that $1{\cdot}4 < D_{20} < 1{\cdot}6$.

The partial specific volume was found to be $V_{20} = 0{\cdot}733$ for the pathological 20-component from β_0 as compared with $V_{20} = 0{\cdot}715$ found by Kabat and Pedersen (1938) for antipneumococcus serum globulin from horse.

From $s_{20} = 19{\cdot}1$, $D_{20} = 1{\cdot}4$ or $1{\cdot}6$ and $V_{20} = 0{\cdot}733$ we have $M = 1{\cdot}24 \cdot 10^6$ or $1{\cdot}08 \cdot 10^6$.

From $s_{20} = 19{\cdot}5$, $D_{20} = 1{\cdot}4$ or $1{\cdot}6$ and $V_{20} = 0{\cdot}733$ we have $M = 1{\cdot}27 \cdot 10^6$ or $1{\cdot}11 \cdot 10^6$.

E. The 'X-protein'.

It seems very probable that the composition of the '*X*-protein' (β-globulin) may vary at least between individuals and perhaps also in the same individual as a consequence of its diet. This implies that the composition and particle weight of the '*X*-protein' are not constant. On p. 32 an estimate of the particle weight gave $\sim 1{\cdot}9 \cdot 10^6$. Some further measurements were made in order to find the order of magnitude of the '*X*-protein' particle.

As long as the '*X*-protein' has not been isolated in a pure condition its partial specific volume cannot be determined. The density of the particle may, however, be estimated, as the variation of s with ϱ is known. From UC eq. 65 we have

$$s = \frac{M(1 - V\varrho)}{f}.$$

If we assume that M, f and V are constant and independent of the concentration of salt in the solution, we have

$$\frac{s_1}{1 - V\varrho_1} = \frac{s_2}{1 - V\varrho_2} \quad \text{or} \quad V = \frac{s_2 - s_1}{s_2\varrho_1 - s_1\varrho_2} .$$

From the line of regression for the isolated β-globulin in Fig. 11 we find $V = 0{\cdot}969$. In this case, where the variation of s with ϱ is so very large, we naturally might just as well have determined the value for ϱ corresponding to $s = 0$ and taken $V = 1/\varrho$. It must be emphasized that V determined in this way gives the specific volume for the whole particle including its hydration.

Two diffusion experiments were carried out on the isolated 'X-protein'. The first was carried out on 'X-protein' (from serum KOP $^{1}/_{12}$ 43) which had been twice separated in the air-driven centrifuge and finally dialysed against 0·2 M. glycine. For D_A was found 1·52 and for D_m 2·2, but the curves showed definitely that the material was far from homogeneous.

The next experiment was carried out with 'X-protein' isolated from another serum (BF $^{16}/_{2}$ 44). It was dialysed against 0·2 M. glycine + + 0·02 M-NaF (the sodium fluoride being added in order to reduce a possible lipase activity). On this occasion rather satisfactory diffusion curves were obtained which gave $D_A = 1{\cdot}68$ and $D_m = 1.82$.

From $s_{20} = 5{\cdot}9$, $D_{20} = 1{\cdot}68$ and $V_{20} = 0{\cdot}969$ we obtain for the hydrated 'X-protein' a particle weight of $2{\cdot}6 \cdot 10^6$. If it be assumed that the particle is spherical, we have a weight of $1{\cdot}9 \cdot 10^6$.

A phosphorus determination was made by Mrs. B. Wikén on the 'X-protein' isolated from the BF-serum. She found 0·069 mg. P/ml. solution; as Δn for the solution was 148, the protein concentration could be estimated as 7·4 mg./ml. The 'X-protein' should then contain 9·3 mg. P/g. protein. According to centrifuge runs not more than half of the protein consisted of the 'labile component'. If we assume that all the phosphorus belongs to the 'labile component', we find as an upper value for this 18·6 mg. P/g. protein. If we suppose that all the phosphorus belongs to the phosphatide, we obtain the two limits for the phosphatide content 219 mg. phosphatide/g. protein and 438 mg. phosphatide/g. protein. These preliminary values are indeed very high and require confirmation (*cf.* Blix *et al.*, 1941, and p. 32).

4. DISCUSSION AND CONCLUSIONS.

The variation of s_{20} with the concentration of the sedimenting substance has been determined for several of the somewhat purer protein fractions prepared from serum. A very pronounced decrease was found for s_{20} with increase in Δn (determined from the sedimentation diagrams). *Cf.* also Fig. 31—34. The smallest decrease observed for a 1 per cent solution was about 6 per cent, while for the 20-components from two pathological human sera it amounted to more than 20 per cent. If the sedimentation constant is to be employed for the calculation of the molecular weight, it follows that it is necessary to extrapolate to s^0_{20} and not to use $s^{1\%}_{20}$ or the value of s_{20} for an undefined dilute solution. There is another advantage in using s^0_{20} in that an approximately correct value for this constant may be obtained even when the solution is not 'ultracentrifugally pure', while $s^{1\%}_{20}$ varies with the purity of the solution.

It was difficult to know in advance whether the variation of s_{20} with c would have the form $s_{20} = a_1 - b_1 \cdot c$ or $s_{20} = a_2 - b_2 \cdot c\,s$. For some of the fractions the lines of regression were determined for both cases, but in all instances it was found that the correlation was better with the first equation.

For some of the fractions the correlation was excellent, while for others, especially fetuin, it was less good. For this protein it may be suspected that some of the values taken from the different tables do not solely represent fetuin. They may have been taken from symmetrical peaks, supposed to belong to fetuin, which in fact represented both fetuin and, for instance, some '*X*-protein' (α- or β-globulin).

The difference found between the values for s_{20}, D_{20} and V_{20} for fetuin from the calf and those from the various fœtus is a little puzzling. It seems, however, that the experimental material is at present too small to permit the drawing of conclusions. It may also be necessary to compare the values for more purified fractions of the different fetuins. It is hoped that the planned experiments in amino acid analysis (*cf.* p. 146) may throw some light on the problem.

The values found for s_{20} are in almost all instances higher than the corresponding ones found in the U.S.A. The reason for this is not clear. For the serum albumins s^0_{20} is also higher than those previously published from this laboratory; the difference may be explained, how-

ever, by the fact that it was earlier assumed that $s^0_{20} = s_{20}$ for albumin solutions with a concentration below 0·5 per cent.

Of the iso-haemagglutinin too little material was prepared and no diffusion measurements or determinations of V_{20} could be made. As, however, its sedimentation constant is very close to those for the pathological euglobulins, but the decrease in s_{20} with increase in the concentration is less than half as great as for the euglobulins, it seems reasonable to suppose that the agglutinin deviates much less from the compact spherical shape than do the euglobulins. Assuming the normal V_{20} for globulin, the molecular weight should lie between $4 \cdot 10^5$ and $10 \cdot 10^5$, probably closer to the former than to the latter.

The partial specific volumes obtained for the serum proteins were in several cases lower than those given in UC Table 48; it seems that some of these old values may be in need of revision.

A summary of the molecular constants determined in the present investigation is given in Table 50, p. 172.

SUMMARY.

In the first chapter of the present investigation, the factors influencing the appearance of the sedimentation diagrams for human serum have been studied and the optimum concentrations of protein and salt have been determined.

It has been found that the position of one of the components in human serum, the '*X*-protein', is highly dependent upon the density of the centrifuged solution. From experiments with various salt solutions, having different densities, the specific volume for the '*X*-protein' particle is calculated to be $V_{20} = 0{\cdot}969$. Its low density has made it possible to separate the '*X*-protein' from the greater part of the other components in serum by high speed centrifugation. The isolated '*X*-protein' shows sedimentation properties similar to those of the 'labile component' in human serum. An electrophoretically prepared serum fraction rich in β globulin behaves similarly in the centrifuge.

From the variation of the concentration of the albumin, globulin and '*X*-protein' with the total protein concentration in serum it has been concluded that the '*X*-protein' is a dissociable complex of albumin and globulin. From other experiences it has furthermore been gathered that the '*X*-protein' contains great quantities of lipids and probably also some carbohydrates. It may be supposed that the '*X*-protein' plays a very important rôle in the transport function of the blood.

In the non-human sera investigated, no labile component has been observed in the sedimentation diagrams, but all experiences from this and other investigations indicate that a similar '*X*-protein' must also exist in animal sera. In these cases, however, it must be supposed that the sedimentation properties of the '*X*-protein' resemble those of the albumin.

Sedimentation experiments with calf serum, known to contain appreciable amounts of a component (fetuin) sedimenting at a rate 30 per cent lower than albumin, have shown that even in such cases the 'albumin' peak appears to be uniform. The presence of the other component may only be suspected from the comparatively low sedimentation constant for the 'albumin'.

The extraction of lipids from serum and serum proteins destroys the '*X*-protein' and denatures large parts of the globulin, but seems to do quite little harm to the fetuin and the albumin.

In the second chapter the fractionation of serum with ammonium sulphate has been studied. The aim has been to find a convenient method for preparing ultracentrifugally pure serum proteins in some quantity and no regard has been taken to those proteins present only in minute amounts.

By successive precipitation of the globulins within rather narrow concentration ranges only the first globulin fraction deposited appears comparatively pure in the ultracentrifuge. All the other fractions are mixtures of two or more components. Repeated re-fractionation of these solutions with ammonium sulphate makes no great changes in the composition of the fractions. Isoelectric precipitation according to the method of Green produces further changes in some of the mixtures, but in no case results in the isolation of a pure component.

Precipitations carried out at *p*H values alternating between 7 and 5 seems to offer certain advantages in the separation of the different components.

The possibility has been discussed of explaining most of the difficulties connected with the fractionation of serum and the preparation of pure serum proteins by the presence of the '*X*-protein' in the various fractions.

Experiments with serum from newly born calves reveals the presence in considerable amounts of a globulin with molecular weight about 50,000. The same protein predominates in the globulin fraction of serum from cow's fœtus. It has been called fetuin. The fetuin has also been found in large quantities in the sera from the foal and from sheep's fœtus. In the sera from the umbilical cord (man) and from rabbit's fœtus it is present in small amounts only. The difference in the occurrence of the fetuin has been related to other biological dissimilarities between the species concerned.

In the third chapter some of the fractions have been studied in sedimentation and diffusion. The variation of the sedimentation constant with the concentration, as measured from the sedimentation diagrams, has been determined for various components in serum. Some of the values found for s^0_{20} are higher than those given in the literature.

The importance of removing the inhomogeneous heavy material,

present in most serum fractions, before investigating the protein solution in diffusion, has been demonstrated.

The molecular weights for various γ-globulins, albumins, fetuins and 20-components have been calculated from new values for s_{20}, D_{20} and V_{20}. The approximate molecular weight for the iso-haemagglutinin and the particle weight for the 'X-protein' have been estimated. A summary of these molecular constants is given in Table 50.

Table 50. Molecular Constants of Some of the Serum Proteins.

Protein	s_{20}	D_{20}	V_{20}*)	M	f/f_0
Fetuins:					
From calf	$3{\cdot}28 - 0{\cdot}0020 \cdot \Delta n$	$5{\cdot}5$	$0{\cdot}71_4$	50,600	1·60
From cow's fœtus	$3{\cdot}09 - 0{\cdot}0015 \cdot \Delta n$	$5{\cdot}0$	$0{\cdot}69_2$	48,700	1·80
do.	$3{\cdot}23 - 0{\cdot}0020 \cdot \Delta n$	(5·5)	$0{\cdot}71_2$	49,500	1·61
Serum albumins:					
Human	$4{\cdot}6_7 - 0{\cdot}0022 \cdot \Delta n$	$5{\cdot}9_3$	$0{\cdot}73_6$	72,300	1·30
From cow's fœtus	$4{\cdot}7_9 - 0{\cdot}0019 \cdot \Delta n$	$6{\cdot}4_9$	(0·736)	67,800	1·21
Globulins:					
γ-globulin from cow:					
$P_{II}\,\delta_1$	$7{\cdot}4_3 - 0{\cdot}0040 \cdot \Delta n$	$3{\cdot}7_4$	(0·732)	180,000	1·52
$g_1\,\delta_7$	$7{\cdot}6_1 - 0{\cdot}0025 \cdot \Delta n$	$3{\cdot}5_8$	$0{\cdot}73_2$	192,000	1·56
$g_1\,\delta_8$	$7{\cdot}6_5 - 0{\cdot}0029 \cdot \Delta n$	$3{\cdot}8_1$	(0·732)	182,000	1·49
Human γ globulin	$7{\cdot}1_2 - 0{\cdot}0034 \cdot \Delta n$	$4{\cdot}0$	$0{\cdot}71_8$	153,000	1·51
Anticomplementary human pseudoglobulin	$7{\cdot}1_4$	$3{\cdot}8_0$	$0{\cdot}73_2$	170,000	1·53
20-components:					
Iso-haemagglutinin, human	$19{\cdot}8 - 0{\cdot}0074 \cdot \Delta n$		(0·73)	($\sim 5 \cdot 10^5$)	
Pathological human euglobulin					
From β_7	$19{\cdot}1 - 0{\cdot}022 \cdot \Delta n$	$>1{\cdot}4$	$0{\cdot}73_3$	$\sim 1{\cdot}2 \cdot 10^6$	2·1
From β_9	$19{\cdot}5 - 0{\cdot}022 \cdot \Delta n$	$<1{\cdot}6$	(0·733)	$\sim 1{\cdot}2 \cdot 10^6$	2·0
'X-protein':					
Human	5·9	1·7	$0{\cdot}96_9$†)	($\sim 2{\cdot}10^6$)†)	

*) Determinations by C. Drucker. †) For the hydrated particle.

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CONTENTS.

Page

Preface 5

Introduction 7

Chapter I. Ultracentrifugation of Serum 12

1. Historical 12
2. General 14
   - A. Selection of Experimental Conditions 15
   - B. Determination of the Amounts of the Different Components from the Sedimentation Diagrams 15
   - C. Influence of Concentration 19
   - D. Influence of Salts 23
   - E. Discussion and Conclusions 31
3. Ultracentrifugation of Different Sera and Plasmas 33
   - A. Human Serum 33
     - a. Normal Human Serum and Plasma from Individuals Belonging to Different Blood-groups 34
     - b. Pathological Sera 39
   - B. Other Sera 40
     - a. Sera from Calf and from Cow's Fœtus 41
   - C. Extraction of Lipids from Serum and Serum Proteins . . . 43

Chapter II. Fractionation of Serum 52

1. Historical 52
2. General 57
   - A. Choice of Salt Concentration Range for Fractionation 61
3. Fractionation of Different Sera 68
   - A. Cow Serum 69
     - a. Adult Cow Serum 69
     - b. Calf Serum 85
     - c. Fœtal Serum from Cow 109
   - B. Human Serum and Plasma 116
     - a. Normal Adult Human Serum and Plasma 116
     - b. Pathological Human Serum 121
     - c. Serum from the Umbilical Cord 125

Page

C. Serum from other Species 125
a. Adult and Fœtal Serum from Rabbit 125
b. Foal Serum 127
c. Fœtal Serum from Sheep 128
D. Fractionation of Delipidated Serum 128
a. Calf Serum 129
b. Human Serum 133
4. Discussion and Conclusions 135

Chapter III. Measurements on Individual Serum Proteins 148

1. Historical 148
2. General 151
3. Examples 152
A. Fetuins 153
B. Serum Albumins 157
a. Human Serum Albumins 157
b. Serum Albumins from Cow's Fœtus and from Calf . . . 158
C. Serum Globulins 159
a. γ-Globulin from Cow 159
b. Human γ-globulin 161
c. Other Globulins 162
D. The 20-Components 163
E. The '*X*-protein' 166
4. Discussion and Conclusions 168

Summary 170

References 173